Perinatal Lead (Pb) Exposure's Effect on DNA Methylation and Hydroxymethylation and Risk of Adverse Neurodevelopmental Outcomes

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Environmental Health Sciences)
in the University of Michigan
2021

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Dedication

This dissertation is dedicated to my grandmothers, Shirley A Wenzel and Patricia A Rygiel, who both ascended into heaven while I was pursuing this doctoral degree. They empowered me as a young woman to pursue the path laid out by God, because with His guidance anything is possible. They taught me how rare and beautiful it truly is to even exist.

Acknowledgements

I would like to start by thanking my primary research mentor, Dr. Dana Dolinoy, for her continued support and guidance throughout my PhD program. She was also so very generous with her time, knowledge, and patience. As a result of her expertise, I gained a much deeper understanding of environmental health, toxicology, and exposure science. My gratitude for her contribution to my future success is immeasurable. I would also like to thank my committee members, Dr. Maureen Sartor, Dr. Justin Colacino, Dr. Jaclyn Goodrich, and Dr. Kelly Bakulski. They all lent me their time, expertise, and patience. Without their assistance, I would still be struggling with R code, Linux, and bioinformatics.

I am grateful for my funding sources that allowed me to focus on my research training – Genome Science Training Program (GSTP), Rackham Pre-candidate and Candidate Research Awards, Children's Environmental Health Center, CHEAR Program, and Michigan Lifestage Environmental Exposures and Disease (M-LEEaD). Without their support, none of the data would have been collected and this dissertation would not be plausible. I would like to acknowledge the ELEMENT study (Early Life Exposures in Mexico to Environmental Toxicants), the Mexican Social Security Institute, and the mothers and infants who participated, and continue to participate, in this multigenerational project.

I would like to acknowledge all the members of my PhD cohort and the Dolinoy Lab for their physical and emotional support over the years. Specifically, our lab

manager, Tamara Jones, for all her guidance with perfect lab techniques, and I mean perfect aseptic techniques. Dr. Goodrich for introducing me to the lab before I was a student and getting me acquainted with lab protocols, R code, and the ELEMENT cohort. She also deserves a special thanks for always being able to edit every manuscript I have written. Future Dr. Ziying (Nancy) Lei for always surprising me with yummy treats at my desk. Dr. Pinithi Perera, Dr. Joseph Kochmanski, and Dr. Kari Neier, for being the best 'upperclassmen' mentors a precandidate could ask for. You three not only answered all my annoying questions, but you also made me critically think through it all. I needed your push in order to succeed. Last, but certainly not least, Kat Springer, an undergraduate student who helped me organize majority of the beautiful tables within this dissertation.

A special shout out to my AP Chemistry teacher, Yasmeen Youngs, who introduced me to science in an exciting, engaging way through the Rainbow Connection. At the moment, I was hooked.

I would like to thank my parents, Larry and Nancy, for providing unconditional love, support, and a listening ear. Their encouragement and guidance, even when they didn't understand the science, was greatly needed and appreciated. My brothers, Andrew and Michael, for always reminding me I have been in school 'forever,' but also bragging to their friends about how smart their sister is. For my friends in Ann Arbor, greater Detroit, at Mt. Holly, and in Colorado, for keeping me sane. I would like to thank my dog, Kiowa, for reminding me to just get outside and play when things get ruff. The great outdoors calms the soul. Finally, Dr. Reid K. Smith, my partner, who has provided me with many hugs, taken me on many adventures, expanded my movie viewing and

music listening, introduced me to mountain biking, and delivered unwavering love for me and my dreams.

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Abstract

The health impacts of prenatal Pb exposure can be framed with the Developmental Origins of Health and Disease (DOHaD) theory, which postulates that exposure to environmental factors (i.e., diet, chemicals, stress, etc.) during sensitive periods of development (i.e., pre-conception, gestation, neonatal, etc.) alters an organism's molecular biology, physiology, and metabolism, potentially leading to myriad effects on cognition, growth, and maturation. Around the world, exposure to lead (Pb) continues to be a persistent problem. A particularly vulnerable population includes pregnant women, where Pb from both current and past exposures can affect their developing fetuses. Disruptions in normal epigenetic processes during gestation impact gene regulation and subsequent outcomes. Accumulating evidence for DOHaD includes potential mechanisms such as epigenetic reprogramming via DNA methylation (5mC) and, less studied, DNA hydroxymethylation (5hmC), linking developmental exposures to later-life outcomes. This dissertation aims to examine whether Pb exposure influences DNA 5mC and 5hmC and susceptibility to early cognitive delays.

A human longitudinal cohort, Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT), was used to investigate whether developmental Pb exposure would alter epigenetics at birth and in adolescence, and if these alterations are associated with decreases in neurodevelopmental scores in early infancy. The first aim of this dissertation characterized the association between prenatal Pb exposure measured in maternal blood and bone and CpG site-specific DNA methylation at

>750,000 loci at birth in infant umbilical cord blood cells. Linear regression models were used to identify differentially methylated CpG sites by prenatal Pb exposure, using the false discovery rate method to correct for multiple testing. In the second aim, we evaluated both gene-specific DNA 5mC and 5hmC in adolescent whole blood from the same cohort to determine whether the association of prenatal Pb exposure and epigenetics persisted into adolescence and determine if 5hmC is associated with the exposure. Finally, in the third aim we assessed whether DNA methylation at birth mediates the association between prenatal Pb exposure and adverse neurodevelopmental outcomes at 12- and 24-months of age.

In Aim 1, we found that trimester-specific developmental Pb exposures were associated with gene-specific DNA methylation changes at birth, and most changes were identified in genes related to neurodevelopment. Next, we showed that some of these Pb-induced DNA methylation changes were stable into adolescence, particularly those associated with first trimester exposure. This potentially suggests that perturbations occurring during the first trimester are reprogramming the epigenome and these changes remain into adolescence. Within Aim 2, we observed widespread, variable 5hmC levels in adolescent whole blood. We also revealed that prenatal Pb exposure has independent associations with both 5mC and 5hmC. These associations were also sex-specific at some loci, indicating a sexually dimorphic effect on the epigenome. Finally, the Aim 3 mediation analysis showed DNA methylation mediated the association between second trimester Pb exposure and cognition scores at 24-months of age at multiple neurodevelopmental measures.

Overall, this dissertation illustrates that prenatal exposures to Pb can modulate epigenetics, and this may be one mechanism linking exposure to persistent effects on development and offspring health. Additional studies in larger cohorts or animal studies are needed in order to confirm these findings. These results emphasize the importance of distinguishing between 5mC and 5hmC during epigenetic studies, thus presenting a new conceivable mechanism describing the underlying biology behind the association between exposure and outcome, 5hmC.

Chapter 1 Introduction

Childhood Lead (Pb) Exposure and Public Health Epidemiology

The metal lead (Pb) is an element that has been used for thousands of years due to the ease of extraction from ores, relative abundance on earth, and low cost [1]. Pb is a ubiquitous environmental pollutant with potential sources from industries, mining and smelting, leaded gasoline, lead-based paint, watering piping, and consumer products (such as toys) that use Pb. The most common sources of human exposure include paint, dust, water, or soil through ingestion, inhalation, and skin absorption [2-4]. Pb's inorganic forms have favorable absorption, distribution, and excretion related to respiratory and digestive poisoning, whereas organic Pb is easily absorbed through the skin [5]. Blood lead levels (BLLs) within the US population has fallen dramatically, 93.6% on average from 1976-1980 to 2015-2016, since the phasing out of leaded gasoline and bans of Pb-based paint, and canned foods and other consumer products with Pb solder [6, 7]. These results suggest that public health reforms targeting Pb exposure were largely successful. However, concern has now grown regarding chronic low-level exposure within the environment, such as in dust from older homes with Pb-based paint and aging infrastructures where Pb leaches from old pipes into the water source [8-10]. It is estimated that roughly 800 million children worldwide have blood Pb concentrations greater than 5 μg/dL with the US having nearly 1.23 million children above this limit and approximately 159,000 over 10 μg/dL [11]. The incidence of Pb poisoning is associated with numerous factors, including socioeconomic status, rurality, race, age, and the

dates one's residence was built. In low- and middle-income countries, Pb exposure occurs through various contamination sources such as use of Pb-glazed ceramics and Pb-acid battery production and recycling [12].

The number of children exposed annually to Pb in the U.S. and globally is astonishing, nearly 800 million globally [11] and accounting for 0.6% of the global burden of disease with most of the exposures affecting children [1]. Worldwide Pb exposure is responsible for 24.4 million disability-adjusted life years from intellectual disability [13]. Cognitive delays can compromise school performance and increase aggressiveness, making Pb poisoning a public health concern. It is a natural toxicant that can adversely affect nearly every organ, particularly the brain resulting in neurodevelopment impairments. Most research has been examining toxicity at high levels of exposure, but more recent research has focused on understanding the impact of Pb exposure at or around the blood Pb reference of <10 μg/dL or <5 μg/dL, especially within pregnant women and children. Pb exposure is more toxic on developing brains in children than on mature brains, even at low BLLs, highlighting the importance of reducing exposures among pregnant women and young children [14, 15]. There is no known physiological role of Pb in the body and there is no known level of Pb that is considered safe.

While environmental health scientists have long known of the adverse neurological effects of high levels of Pb exposure, epidemiological studies continue to demonstrate that Pb exposure affects cognition and behavior with major impacts on proper functions, including academic development even at very low levels of exposure. A meta-analysis of 1,333 children (233 with BLLs ≤10 μg/dL and 103 ≤7.5 μg/dL) found an inverse relationship between ln-transformed BLLs and cognition using the

intelligence quotient compromise, estimated at a 3.9 point decrease when levels rise from 2.4 to 10 μg/dL [14]. Interestingly, the BLLs <7.5 μg/dL showed greater loss of intelligence quotient points per unit increase in Pb compared to the BLLs at or above 7.5 μg/dL providing data on low dose neurocognitive effects. Another study analyzed data from 534 children and found that BLLs <10 μg/dL had significant decreases in intelligence and academic achievement, specifically 5.0 points lower intelligence quotient scores when comparing those with exposures 5-10 μg/dL to children with lower BLLs of 1-2 μg/dL [16]. Negative associations between Pb and multiple cognitive measures were even observed with exposures as low as 3 μg/dL. In addition to intelligence quotient, numerous studies have suggested that Pb exposure contributes to the incidence of behavioral disorders [14, 17-19]. Specifically, a study comprising 294 children found that BLLs at 12 months of age were significantly associated with weaker psychomotor development at 24 months of age, which measures attention and processing speed [18]. Another study found that children with BLLs as low as 3 μg/dL exhibited deficits in basic reaction time, fine motor skills, and attention [19]. Each study concludes that there is no apparent safe lower threshold for postnatal Pb exposure, and while we understand the gross effects of Pb on cognition, further research is needed to more comprehensively understand the biological mechanisms that underlie Pb-related adverse impacts on cognitive development.

Fetal Pb Exposure and Developmental Origins of Health and Disease

In addition to childhood Pb exposure, fetal Pb exposure is of great public health concern. Fetal Pb exposure is associated with developmental and cognitive deficits, many of which are long-lasting. This is line with the Developmental Origins of Health

and Disease hypothesis which states that exposures to nutritional and environmental factors during prenatal and early postnatal periods impact health outcomes into adulthood [20-23]. This theory stems from the idea that the fetal environment primes the organism for the environment in which they will soon live, where perturbations during these early critical periods can result in reprogramming of cells and tissues that influence susceptibility to disease. There is strong evidence for DOHaD with respect to Pb exposure. Research into the Developmental Origins of Health and Disease has shown that perinatal exposure to Pb increases susceptibility to cognitive deficits later in life. Permanent cognitive and behavioral changes have been documented with early-life Pb exposure [18, 24, 25].

Epidemiological studies of Pb exposed pregnant women have reported BLLs below 5μg/dL can be associated with disturbances in early mental growth and later intellectual function including negative temperament and dramatic IQ point decreases. Specifically, gestational exposure measures in mothers below 5 μg/dL [26] and in cord blood below 2 μg/dL [27] have been associated with decreased neonatal and infant behavioral neurological scores utilizing the neonatal behavioral neurological assessments and Bayley Scales of Infant Development II Mental Development Index, respectively. Another study provided evidence for lower Mental Development Index scores as early as 6 months of age due to late pregnancy maternal Pb exposures below 5 μg/dL [28]. Lastly, the Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) maternal-offspring pregnancy and birth cohort utilizes a series of longitudinal birth cohorts to investigate the influence of Pb exposure – *in utero* and in childhood – on sensitive periods of development. They found that first trimester

maternal BLLs are associated with decreases upwards of 6.94 points in 24-month-old offspring per log microgram per liter Pb using the BSID-II Mental Development Index [24]. Mothers were recruited during prenatal visits and samples were collected at each trimester and offspring continue to be followed to collect vast amounts of data on biological, psychological, and physical measures. The current dissertation will take advantage of the available biological samples and neurodevelopmental measures from ELEMENT to expand on this research study. Despite major advances to reduce environmental exposures, epidemiological studies continue to demonstrate that Pb exposure affects cognition and behavior with major impacts on proper neurological functions [14, 17, 29, 30].

Pb Exposure Affects Early-Life Neurotoxicity

The development fetus is particularly sensitive to environmental contaminants, including Pb [31]. The fetus can be exposed by stored Pb in bone from the mother's previous exposures nearly decades prior as well as concurrent Pb exposure circulating in the blood. The half-life of Pb, which is documented to be about 40 days in blood, results in its lingering presence in the bloodstream after initial exposure [32]. Once in the bloodstream, it distributes throughout the body and accumulates in bone. Pb forms highly stable complexes with phosphate that can replace calcium in the calcium-phosphate crystalline matrix of bone [33]. Residence time in bone ranges from several years to decades [34], accounting for more than 90% and 50% of total body burden of Pb in adults and infants, respectively [35]. Physiological states, such as pregnancy, menopause, advanced age, and diseases states, such as osteoporosis, are associated with increased bone resorption to blood [36, 37].

Pb is known to be released into blood from bone during pregnancy and becomes a source of exposure *in utero* [36]. Further, early life exposure can occur via placental transfer and in infancy through breast milk. Bone stores the highest content of Pb, and as bone resorption increases during pregnancy, this consequently releases Pb into the bloodstream, from where it could prospectively reach the developing fetus potentially causing abnormalities in fetal development [37, 38]. The placenta does not provide an effective barrier against Pb [39, 40]. Therefore, it could cross the placenta barrier, disrupt the blood-brain barrier, and disturb brain development and architecture by interfering with hardwiring and ongoing neurotransmission during development *in utero.* Neurotransmitters are chemicals used by neurons to send signals to other cells, Pb can disrupt communication between cells by affecting neurotransmitter storage and release and altering receptor density [25, 41].

In addition to disrupting neurotransmission, there are several other known mechanisms of Pb neurotoxicity. Pb competes with Ca^{++} and disrupts its homeostasis, substitutes for zinc, alters lipid metabolism, can accumulate in astrocytes, and causes mitochondrial damage through various avenues such as opening pores, damaging membranes, stimulating release of Ca++, which results in apoptosis [41, 42]. When Pb alters the release of neurotransmitters from presynaptic nerve endings, spontaneous release is evoked and release is inhibited resulting in disruption of neuronal activity, thus, altering the developmental processes of synapse formation [43]. These processes result in damage to the central nervous system, potentially causing loss of memory and vision, and lowered cognitive and behavioral abilities leading to brain damage and/or neurodegeneration later in life. Studies have shown that developmental Pb exposure in

rats decreases hippocampal long-term potentiation, which is necessary for learning and memory [44-46]. This study provided evidence that memory and spatial learning are vulnerable to impairment by Pb because Pb damages the developing hippocampus. Thus far, there is epidemiological evidence for the link between prenatal Pb exposure and evidence for several biological mechanisms by which Pb exerts its' effects. Recently, evidence for an addition mechanism by which Pb may impart long-term effects on children is emerging: epigenetic regulation.

Epigenetics and the Distinct Differences between DNA Methylation (5mC) and Hydroxymethylation (5hmC)

One way in which developmental exposures impact infant and adult disease status is through alterations in gene regulation via epigenetic modifications, such as DNA methylation, histone modifications, and non-coding RNA interactions [47-50]. Epigenetics is the study of mitotically heritable and potentially reversible changes in gene expression that are independent of DNA sequence. DNA 5mC is the addition of a methyl group covalently bound to the 5'-carbon of Cytosine (**Figure 1.1**); in mammals this typically occurs on a Cytosine adjacent to a Guanine, referred to as a CpG site [51, 52]. These CpG sites are normally found near promoter regions, exon junctions, and enhancers, which influence gene expression [53]. Increased levels of 5mC are associated with decreased transcription factor binding at promoter/enhancer sites and suppression of transcription [54]. DNA methyltransferases add the methyl groups and if DNA methyltransferases fail to add new methyl marks during cellular replication, DNA can become demethylated, which is termed passive demethylation or loss of methylation (**Figure 1.1**) [55, 56]. Alternatively, active DNA demethylation can also occur when the

methyl group is oxidized by TET enzymes into hydroxyl group (5hmC) followed by formyl (5fC) and carboxy (5caC) groups that are removed by base excision repair pathways and targeted demethylation (**Figure 1.1**) [57-59]. Active demethylation involves the activity of thymine DNA glycosylase-mediated base excision repair, which only recognizes 5fC and 5caC and removes them, thus restoring unmethylated cytosines (**Figure 1.1**). These intermediates have a short half-life while 5hmC is believed to be a more stable modification of cytosine [58, 60-62].

The role of 5hmC as an important epigenetic mark of active DNA demethylation remains a great debate [63]. Genome-wide 5hmC is associated with increased gene expression in human and mouse embryonic stem cells, suggesting a role in transcriptional control [64, 65]. 5hmC mediates the binding affinity of proteins that recruit 5hmC-binding proteins by acting as an intermediate for oxidative demethylation or a stable modification that eliminates the need for the removal of the methyl groups [66-68]. Global 5hmC is highest in the brain and is also present in the liver, testes, placenta, colon, and blood, though in progressively lower levels [67, 69-73]. In several human and animal studies, early-life neurologic disorders and later-life neurodegenerative disorders have been associated with 5hmC perturbations in the brain [74-77]. Unfortunately, most current methods, including the gold standard sodium bisulfite sequencing, collectively measure 5mC and 5hmC without distinguishing between the two. Detecting changes in levels of 5hmC by exposures such as Pb may provide additional insights into mechanisms related to neurodevelopment toxicity.

Epigenome and the Environment

The epigenome responds to environmental exposures. Environmentally sensitive regions of gene regulation can influence long-term epigenetic reprogramming [48, 50]. There is evidence in vitro, humans, and animal studies that Pb can alter epigenetic regulation (**Tables 1.1 & 1.2)**. One study exposed rat pheochromocytoma cells (PC12) to three concentrations of Pb (50, 250 and 500 nM) and assessed DNA methylation patterns in amyloid precursor protein (APP), which has been associated with the pathogenesis of Alzheimer's disease, and *DMNT1* [78]. They showed that exposure at all three doses were associated with hypomethylation in the *APP* promoter, and 500 nM group had global methylation and *DNMT1* expression changes as soon as 2 days post-exposure. Another study investigated the effects of various Pb concentrations (0.4μM, 0.8μM, 1.2μM, 1.5μM, and 1.9μM) on neuronal differentiation in human embryonic stem cells (hESCs). Interestingly, Pb exposure did not prevent hESCs from generating neural progenitor cells [79]. Pb induced rapid changes in global undifferentiated and differentiated hESCs DNA methylation in a dose-dependent manner. These studies provide evidence for potential neurotoxic effects through mechanisms altering both global and promoter methylation patterns.

Human epidemiological studies have also identified DNA methylation associated with Pb exposure (**Table 1.1).** A U.S. prospective human pregnancy cohort, Project Viva, with an average prenatal Pb exposure of 1.22 ± 0.63 μg/dL in erythrocytes of pregnant mothers, conducted an epigenome-wide analysis on umbilical cord blood samples (n=268) to evaluate the association between maternal Pb exposure and DNA methylation [80]. They identified sex-specific differentially, mainly hypomethylated, CpG

sites associated with increased Pb; in sex-stratified analyses, more inverse relationships were found between Pb and DNA methylation while the reverse was found among males. Another U.S. study utilizing samples from the Healthy Families Project in southeast Michigan measured at-birth Pb and DNA methylation in dried blood spots (n=96; mean Pb: 0.78 μg/dL), and identified similar associations of mostly hypomethylation with increasing Pb exposure in gene pathways association with development and neurological functions [81]. Another Michigan study measured Pb and DNA methylation profiles in dried blood spots of males (n=25) and females (n=18) 3 months to 5 years of age who reside in Detroit, where nearly half the children had BLLs at or above the Centers for Disease Control and Prevention BLL of concern (5 μg/dL) [31]. Their pilot study identified conserved (irrespective of sex) loci of Pb-associated methylation, as well as female- and male-specific DNA methylation alterations related to Pb exposure. The Early Autism Risk Longitudinal Investigation (EARLI) U.S.-based cohort study investigated prenatal Pb (mean: 0.4 μg/dL) with DNA methylation in maternal first or second trimester whole blood (n=97), and identified global hypermethylation associated with increasing Pb but also site-specific hypermethylation of genes related to nervous system development, and calcium ion binding [82]. Lastly, another study within the ELEMENT cohort (n=247) identified differential *IGF2* hypermethylation in umbilical cord blood with maternal patella bone Pb measures (mean Pb: 14.4 μg/g) and *HSD11B2* hypermethylation among girls while conducting their candidate gene analysis [83]. These studies found modest evidence for the association between prenatal Pb and DNA methylation, sex- and CpG-site specific, but one limitation encompasses each of these studies: assessment of neurodevelopment.

None of these studies evaluated the potential link between DNA methylation and neurodevelopment, nor did they assess the probability of DNA methylation playing a mediating role in the associations between prenatal Pb and adverse neurodevelopmental outcomes.

In animal research studies (**Table 1.2**), differential methylation by Pb exposure was also identified. For example, a few studies utilized a murine model were post-pubertal virgin *a/a* females, genetically invariant mice 93% identical to C57BL/6J strain, were prenatally exposed to Pb in water at three levels of human physiologically relevant doses two weeks prior to mating with A^{vy}/a, and exposure in the females lasted through lactation. Exposure groups were 2.1 ppm, 16 ppm, and 32 ppm with BLL ranges (mean) of 2.0-5.88 µg/dL (4.1), 13-40 µg/dL (25.1), and 16-60 µg/dL (32.1), respectively. DNA total methylation was assessed via pyrosequencing at four Intracisternal A particle (IAP) elements in the brain of 10 month mice [84]. IAPs are a class of murine retrotransposons that are environmentally responsive [47]. Prenatal Pb exposure reduced DNA methylation around 2-3% at three of the intracisternal A particles in the brain with dose-dependent and sex-specific effects compared to control mice. Another candidate gene analysis assessing A^{vy} and IAP ($Cabp^{IAP}$) with PND21 tail tissue identified hypermethylation at the 2.1ppm Pb levels, but there was hypomethylation at the higher levels, where the sex-effects were driven by males [85]. A genome-wide analysis of 10 month aged cortex nuclei of the mice from the same exposure model identified only hypomethylated CpG sites within each exposure group [86]. Two studies looking at the association between high prenatal Pb exposure (32ppm) in blood and liver DNA methylation at PND21 [87] and

5 months of age [88] identified thousands of tissue- and sex-specific differentially methylated sites and regions with very little overlap between the tissues.

One possible mechanism of Pb-induced changes in DNA methylation levels is through induction of active DNA demethylation. Metal exposures, such as Pb, cause indirect reactive oxygen species formation with thiol depletion resulting in oxidative stress [89, 90]. Mechanistically, oxidative DNA damage is known to inhibit the ability of DNA methyltransferases to interact with DNA, causing hypomethylation at CpG sites [91, 92]. Subsequently, Pb-induced oxidative stress results in the accumulation of α-ketoglutarate [93], a co-factor for TET enzymes which are involved in the oxidation of 5mC to 5hmC [94, 95]. Thus, Pb may increase activity of TET enzymes and increase 5hmC across the genome [94]. Thus, long-term exposure to Pb-induced oxidative stress could result in oxidative damage of methylated cytosines and decrease the level of 5mC while subsequently increasing 5hmC (**Figure 1.1**). Despite the great progress over the years, there are still gaps in animal and human studies regarding where perinatal Pb exposure-mediated epigenomic changes in 5mC and 5hmC occur and whether these are related to adverse neurodevelopmental outcomes.

ELEMENT Cohort Overview

The aims within this dissertation utilize samples from a well-established longitudinal birth cohort, ELEMENT [96]. The ELEMENT Project is a mother-child pregnancy cohort that began in the mid-1990s in Mexico City, Mexico. It consists of three cohorts where data was collected from 1994-1995, 1997-2000, and 2001-2003, respectively. ELEMENT was originally set up to explore two main topics: 1) determine whether fetal growth and neurodevelopment are at risk from enhanced mobilization of

Pb during pregnancy from maternal bone, and 2) determine whether bone lead mobilization during pregnancy and lactation can be suppressed by maternal calcium supplementation and whether the calcium can mitigate adverse effects on offspring health and development. Since then, ELEMENT has become a major research resource on early-life exposures and developmental outcomes, and we continue to study the long-term consequences of toxicant exposures during perinatal period on adolescent children and young adults, some of whom have been followed for over 20 years. We hope to eventually study intergenerational effects of *in utero* exposures. This dissertation utilizes data and biological samples from Cohorts 2 and 3 that contain all the data needed in order to answer each aim's hypothesis.

Dissertation Overview

This project utilizes a human birth cohort to study the underlying mechanisms of the association between prenatal Pb exposures with adverse neurodevelopmental outcomes. The overarching hypotheses for this dissertation are as follows: **1. Developmental exposure to Pb will result in alterations of DNA methylation (5mC) and hydroxymethylation (5hmC) at birth and these changes will persist into adolescence; 2. Epigenetics mediates the association between prenatal Pb exposure and adverse neurodevelopmental outcomes as early as infancy.** To test these hypotheses, Aim 1 uses maternal trimester-specific human whole blood samples and matched infant UCB to identify genome-wide DNA methylation changes associated with prenatal Pb exposure. We expected to see a majority of the changes in methylation to be associated with the first trimester exposure. Further, we expected to see more hypomethylation by Pb genome-wide. Aim 2 expands from the first aim to assess

persistence of associations and also associations between Pb and 5hmC. Matched offspring human blood samples were collected in adolescence to determine if those significant changes in DNA 5mC and 5hmC by prenatal Pb exposure, measured with target gene pyrosequencing, will persist at this later timepoint in neurocognitive-related candidate genes. We expected the ability to detect 5hmC in human whole blood but at low levels (~1-5%). We anticipated the epigenetic reprogramming to persist into adolescence, particularly the changes related to first trimester exposure. Further, we predicted that as 5hmC increased, 5mC would decrease, and prenatal Pb exposure would have associated effects with each. Aim 3 uses mediation modelling to assess whether DNA methylation changes at birth in human umbilical cord blood may mediate the association of trimester-specific Pb exposure and adverse early-life neurodevelopmental indicators. We projected that DNA methylation would indeed mediate the association between prenatal Pb exposure and neurodevelopmental outcomes, and these mediating effects would be noticed as early as 12-months of age. This dissertation generated data necessary for better understanding of prenatal health risks surrounding developmental Pb exposure. This dissertation aims to fill knowledge gaps and contribute to the idea that epigenetics plays a key role in the adverse health outcomes by prenatal Pb exposure (**Figure 1.2**).

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Tables and Figures

Table 1.1: Epidemiological studies showing prenatal and childhood Pb exposure and associations with DNA methylation.

Reference	Region	DNAm timing, tissue, and analysis	Biomarkers used and Pb levels (mean (SD))	Timing of exposure assessment	Findings
Sen et. al.[97]	USA	3 mos to 7 yrs Dried blood spot 450K array	Dried blood spot: 7.78 (6.91) µg/dL	Childhood	Pb influences DNA methylation patterns in a gender-dependent manner.
Goodrich et. al.[83]	Mexico	Neonates UCB Pyrosequencing	Maternal tibia: 9.82 (9.45) µg/g; Maternal patella: 14.4 (14.8) µg/g; UCB: 6.49 (3.52) µg/dL;	Prenatal and at birth	DNA hypermethylation in genes such as *IGF2* in neonates related to ≥10 µg/g in maternal patella bone.
Rygiel et. al.[98]	Mexico	Neonates UCB EPIC array	T1: 6.56 (5.35) µg/dL; T2: 5.93 (5.00) µg/dL; T3: 6.09 (4.51) µg/dL; Maternal patella: 11.8 (9.25) µg/g; Maternal tibia: 11.8 (6.73) µg/g	Prenatal	DNA hypomethylation with increasing Pb at multiple CpG sites. DNA hypermethylation with increasing Pb at the regional level
Montrose et. al.[81]	USA	Neonates Dried blood spot EPIC array	Dried blood spot: 0.78 (0.85) µg/dL	At birth	Majority (82%) of statistically significant CpG sites exhibiting hypomethylation with increasing Pb exposure.
Aung et. al.[82]	USA	T2 or T3 Whole blood 450K	Maternal T2/T3: 0.4 (1.6) µg/dL	Prenatal	Hypermethylation at 11 DNA methylation sites associated with Pb. 50.9-55.8% hypermethylation globally with covariate or surrogate models.
Wu et. al.[80]	USA	Neonates UCB 450K	Maternal RBC: 1.22 (0.63) µg/dL	Prenatal	Low-level Pb exposure associated with DNA hypomethylation. Sex-stratified analysis identified more hypermethylation in males but hypomethylation in females.

Note: DNAm, DNA methylation; mos, months; yrs, years; 450K, Human Methylation 450K Bead chip; UCB, umbilical cord blood; EPIC, Human MethylationEPIC 850K Bead chip; T1, first trimester; T2, second trimester; T3, third trimester; RBC, red blood cells

Table 1.2: Experimental studies of Pb exposure showing DNA methylation effects.

Reference	Experimental model	Exposure time	Timepoint and/or tissue of analysis	Dose/concentration of Pb and route	Findings
Li et. al.[78]	PC12 cells	2 and 7 days	2 and 7 days	50, 250 and 500 nM (0.0103, 0.0517, 0.103 ppm)	Hypomethylation of the *APP* promoter. Changes in the levels of global methylation and expression of *DNMT1*
Bihaqi et. al.[99]	Monkey	Birth to 400 days of age	Brain; 6, 12, and 23 years	1.5 mg/kg/day (1.5 ppm/day) Infant formula	Reduction in levels of proteins involved in DNA methylation and histone modification
Faulk et. al.[85]	Mice	2 weeks prior to mating though lactation and weaning	Tail tissue; PND22	2.1, 16, 32 ppm Drinking water	Hypermethylation at the 2.1 ppm and hypomethylation at higher Pb levels; sex-effect driven by males
Montrose et. al.[84]	Mice	2 weeks prior to mating though lactation and weaning	Brain; 10mo	2.1, 16, 32 ppm Drinking water	Prenatal Pb exposure resulted in hypomethylation at three of the IAPs in the brain, and these changes were dose-dependent and sex-specific.
Dou et. al.[86]	Mice	2 weeks prior to mating though lactation and weaning	Cortex neuronal nuclei, 10mo	2.1 and 32 ppm Drinking water	All statistically significant CpGs within each exposure group compared to control were hypomethylated
Wang et. al.[87]	Mice	2 weeks prior to mating though lactation and weaning	Blood and liver; PDN21	32 ppm Drinking water	~1000 differentially methylated cytosines for each tissue- and sex-specific comparison, mostly hypomethylated; hundreds of tissue- and sex-specific differentially methylated regions
Svoboda et. al.[88]	Mice	2 weeks prior to mating though lactation and weaning	Blood and liver; 5mo	32 ppm Drinking water	Thousands of sex-specific differentially methylated cytosines in the blood and liver of Pb-exposed animals; including gnomically imprinted loci
Senut et. al.[79]	hESC	0 to 19 days	47 days	0.4μM (8 μg/dL), 0.8μM (16 μg/dL), 1.2μM (24 μg/dL), 1.5μM (32 μg/dL), 1.9μM (40 μg/dL)	Pb induced rapid changes in the global DNA methylation patterns of undifferentiated and differentiating hESCs in a dose-dependent manner, mostly hypomethylated

Note: PC12, pheochromocytoma cells; hESC, human embryonic stem cells; ppm, parts per million

Figure 1.1: DNA cytosine methylation and demethylation pathway.
Passive demethylation occurs when DNA replicates in the absence of DNA methyltransferases (DNMTs) (blue dashed arrows). Active methylation (red solid arrows) involves DNMTs covalently bonding a methyl group to the 5'-carbon of cytosine. Active demethylation (solid purple arrows) occurs when TETs hydroxylate 5-methylcytosines (5mC) to form 5-hydroxymethylcytosines (5hmC); further oxidation produces 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). Base excision repair (BER) pathways and targeted demethylation can remove 5fC and 5caC. Alternatively, thymine DNA glycosylase (TDG)-mediated BER can restore unmethylated cytosines through active demethylation. 5mC silences regulatory functions, whereas 5hmC is widely debated as having a role in activating regulatory functions (green dotted arrow).

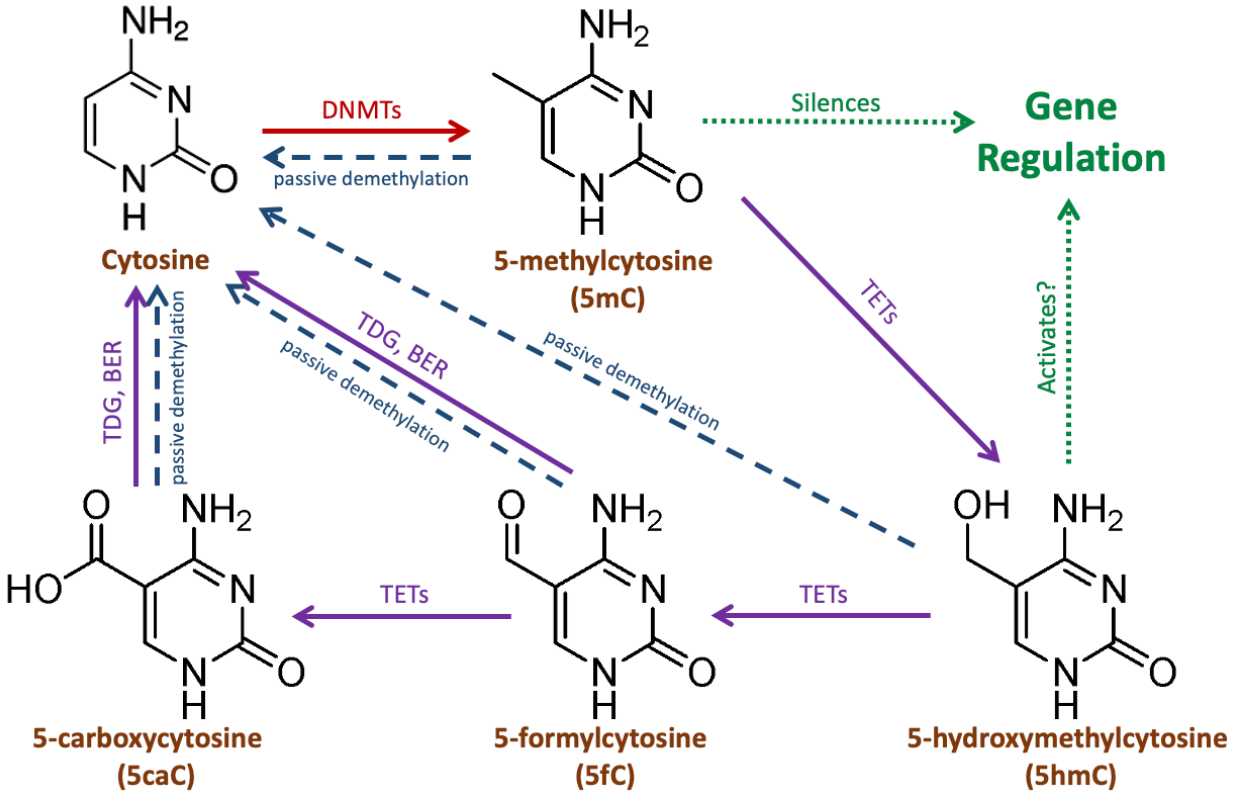
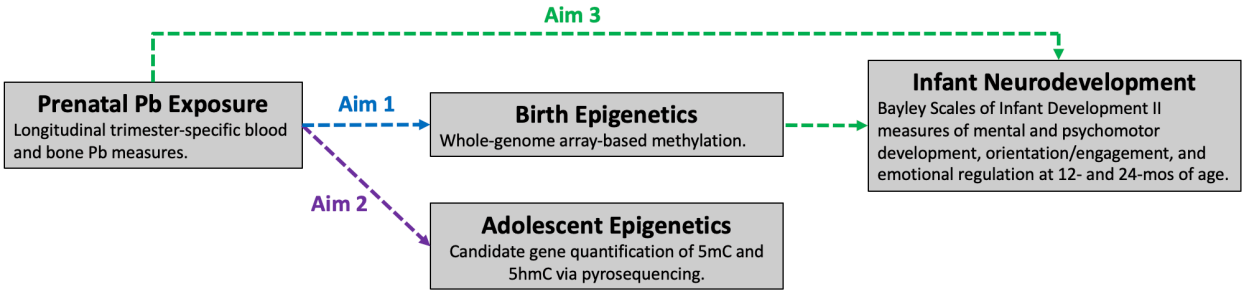


Figure 1.2: Conceptual model summarizing the dissertation aims.
Hypotheses from the three aims work together to examine the effects of environmental factors on epigenetics perturbations and adverse neurodevelopmental outcomes. Each aim is indicated by colors (Aim 1 = blue, Aim 2 = purple, Aim 3 = green). Biomarkers and outcome variables considered within statistical models are indicated within each box.



Chapter 2 Trimester-Specific Associations of Prenatal Lead (Pb) Exposure with Infant Cord Blood DNA Methylation at Birth

Abstract

Gestational exposure to lead (Pb) adversely impacts offspring health through multiple mechanisms, one of which is the alteration of the epigenome including DNA methylation. This study aims to identify differentially methylated CpG sites associated with trimester-specific maternal Pb exposure in umbilical cord blood (UCB) leukocytes. Eighty-nine mother-child dyads from the Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) longitudinal birth cohorts with available UCB samples were selected for DNA methylation analysis via the Infinium MethylationEPIC BeadChip, which quantifies methylation at >850,000 CpG sites. Maternal blood lead levels (BLLs) during each trimester (T1: 6.56±5.35μg/dL; T2: 5.93±5.00μg/dL; T3: 6.09±4.51μg/dL), bone Pb (patella: 11.8±9.25μg/g; tibia: 11.8±6.73μg/g), a measure of cumulative Pb exposure, and UCB Pb (4.86±3.74μg/dL) were measured. After quality control screening, data from 786,024 CpG sites were used to identify differentially methylated positions (DMPs) and regions (DMRs) by Pb biomarkers using separate linear regression models, controlling for sex and estimated UCB cell-type proportions. We identified three DMPs associated with maternal T1 BLL, two with T3 BLL, and two with tibia bone Pb. We identified one DMR within *PDGFRL* associated with T1 BLL, one located at chr6:30095136-30095295 with T3 BLL, and one within *TRHR* with tibia bone Pb (adjusted p-value<0.05). Pathway analysis identified 15 overrepresented gene

pathways for differential methylation that overlapped among all three trimesters with the largest overlap between T1 and T2 (adjusted p-value<0.05). Pathways of interest include nodal signaling pathway and neurological system processes. These data provide evidence for differential methylation by prenatal Pb exposure that may be trimester-specific.

Introduction

Lead (Pb) is a ubiquitous environmental pollutant found in air, soil, water and food. Human exposures mainly occur through ingestion and inhalation, and Pb can be stored long-term in teeth and bones. Pb is a potent neurotoxicant that alters brain development resulting in abnormal cognition and behaviors. Early life Pb exposure also increases the risk for developing a variety of adverse health outcomes later in life, including Alzheimer's, attention-deficit/hyperactivity disorder, cardiovascular disease, and intellectual deficits [1-5]. The Institute for Health Metrics and Evaluation (IHME) estimated in 2017 that Pb exposure accounted for almost 2 percent of total all-cause mortality and at least 2.5 million years of healthy life lost (disability-adjusted life years (DALYs)) worldwide due to idiopathic developmental intellectual disability [6], but this may be an underestimate [7].

The health effects of prenatal Pb exposure can be framed with the Developmental Origins of Health and Disease (DOHaD) theory [8], which links early-life exposures to the development of disease and adverse health effects later in life. The placenta is not an effective barrier against Pb, and since bone reabsorption increases during pregnancy, Pb is released into the bloodstream from where it can reach the fetus potentially causing abnormalities in development [9-13]. The fetus may be exposed to

stored Pb in bone from the mother's previous exposures nearly decades prior as well as to concurrent Pb exposure circulating through the blood.

It has been hypothesized that toxicant exposures, including Pb, may result in epigenetic modifications during sensitive developmental periods, thereby impacting gene regulation and subsequent development and later life health outcomes. Epigenetic modifications, which include DNA methylation, are mitotically heritable and regulate gene expression without altering the underlying DNA sequence. DNA methylation occurs primarily in cytosine-guanine (CpG) dinucleotides, which are enriched in regions called islands, typically located in promoter regions of genes [14,15]. Establishment of DNA methylation patterns occurs in early gestation and disturbances during this period may result in DNA methylation variations that are propagated through mitosis to new cells and developing organs thus affecting gene expression in a wide range of tissues associated with various developmental physiological processes [16].

We and others have provided evidence for the impact of early-life Pb exposure on the epigenome in animal and human studies. In mice, dams were exposed to one of three doses of Pb acetate in water prior to mating through weaning, and the offspring were followed through adulthood and compared to unexposed mice. Lead was associated with altered DNA methylation in offspring tail tips immediately following cessation of exposure (3 weeks of age) at two metastable epialleles[17]. Altered DNA methylation by Pb was also observed in offspring brain (10 months of age) at environmentally-responsive retrotransposons[18]. In a human birth cohort, we quantified DNA methylation at four genes in 247 umbilical cord blood (UCB) leukocyte DNA samples. Biomarkers of prenatal Pb exposure (maternal tibia: 9.82 (SD=9.45) μg/dL,

maternal patella: 14.4 (SD=14.8) μg/dL, UCB: 6.49 (SD=3.52) μg/dL) were associated with percent DNA methylation in neonates in long interspersed elements 1 (LINE-1), as well as growth-related genes (*IGF2* and *HSD11B2*) providing evidence for maternal cumulative Pb burden influencing the epigenome of a developing fetus [19]. Lastly, the U.S. prospective pregnancy cohort, Project Viva conducted an epigenome-wide analysis on 268 UCB samples to evaluate the association between prenatal maternal Pb exposure (mid-to-late gestation Pb in erythrocytes averaged 1.22 (SD=0.63) μg/dL) and DNA methylation [20]. Results identified sex-specific differentially methylated CpG sites by Pb exposure with more found in females (n=38) than males (n=2). Mechanistically, metal exposures such as Pb increase oxidative stress and can cause oxidative DNA damage which inhibits the ability of methyltransferases to bind to DNA; this leads to hypomethylation at some loci [21-23]. Additionally, Pb exposure has been shown to alter expression and function of epigenetic machinery (i.e., DNA methyltransferases (DNMTs)). Several human and animal studies suggest DNA methylation changes from developmental Pb exposure are due to altered DNMT expression and activity [21,24-27].

Despite mounting evidence for Pb's effect on the epigenome, no studies have specifically investigated the association between genome-wide DNA methylation and Pb exposure during each of the three trimesters of pregnancy, as well as cumulative gestational Pb exposure estimated by maternal patella and tibia measures. We hypothesize that prenatal Pb exposure will be significantly associated with changes in DNA methylation patterns across the genome at birth and that these changes will differ depending on the timing of exposure. A whole genome approach will assist researchers

in gaining valuable insight into the effects of prenatal Pb exposure on gene regulation and help to identify potential biomarkers of interest specific to each trimester and cumulative exposure. Here, we performed Pb exposure assessment in multiple biomarkers and DNA methylation profiling in UCB samples from 89 mother-infant pairs enrolled in the Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) project, a longitudinal birth cohort.

Methods

Study population

The Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) project has utilized a series of longitudinal birth cohorts to investigate the influence of exposure to Pb and other toxicants – *in utero* and in childhood – on sensitive periods of development. This project is based on three sequentially recruited cohorts comprising of 1643 mother-infant pairs, some of whom have been followed for over 20+ years [28]. The present study uses data and biological samples from the second and third birth cohorts, for which 1530 women were originally enrolled, and 1012 mother-infant pairs were followed up after birth. Women were recruited from 1997-2000 and 2001-2005, from the Mexican Social Security Institute hospital in Mexico City. Eligibility and exclusion criteria are as previously described [28,29]. For all recruited mother-child pairs, data collected include sex, gestational age, socioeconomic status, anthropometry data, and other environmental exposures. Families were followed-up at multiple timepoints from infancy through adolescent years. For the current study, we selected 97 ELEMENT participants from the second and third cohorts who had archived UCB samples from which we could isolate DNA. The final study comprised of 89 mother-infant dyads whose epigenetic

data passed downstream quality control (QC). Characteristics of these 89 participants compared to all women of cohorts 2 and 3 that had least one Pb biomarker measurement (n=1214) and their children are represented in **Table 2.1A Appendix**.

At the time of enrollment, all mothers were informed about the study; those who agreed to participate read and signed a letter of informed consent about the original study. The original research protocol and all amendments to the study protocol were approved by the Ethics Committees of the National Institutes of Public Health of Mexico, participating hospitals, and the Internal Review Board at all participating institutions including the University of Michigan.

Pb exposure assessment and genomic DNA isolation

UCB was collected within 12 hours after birth. Cohort 2 blood lead levels (BLLs) from each trimester and UCB were measured using inductively coupled plasma mass-spectrometry (ICP-MS, Thermo Finnigan, Bremen, Germany) at the University of California, Santa Cruz, as described previously [30]. Cohort 3 BLLs in maternal venous blood from each trimester and UCB were measured at the Trace Metal Laboratory of the American British Cowdry Hospital using graphite furnace atomic absorption spectrometry (instrument model 3000; PerkinElmer, Norwalk, CT, USA).

Bone Pb was measured in maternal left patella (trabecular bone) and mid-shaft of the left tibia (cortical bone) 1-month post-partum as an indicator of cumulative Pb exposure during pregnancy using a spot-source ^{109}Cd K-shell X-ray fluorescence (K-XRF) instrument. The technical specifications and validation of this instrument is described in detail elsewhere [31]. Analysis of means and standard deviations once weekly for quality control and calibration measures did not disclose any significant shifts

in precision or accuracy. Tibia and patella bone Pb values below the limit of detection (i.e., negative values) were dropped from subsequent analyses resulting in 73 patella bone Pb and 46 tibia bone Pb measures.

DNA was isolated from nucleated UCB cells using Qiagen kits and standard protocols for blood DNA isolation. Nucleic acid yield and purity were assessed first using a NanoDrop spectrophotometer (ThermoFisher Scientific), and doubled stranded DNA was also quantified via a Qubit fluorometer. All DNA samples were stored at -80^{o}C until later use.

Epigenetic analysis

All genomic DNA samples were bisulfite converted (500 ng) using the Zymo EZ DNA Methylation kit (Zymo Research) and the recommended incubation conditions and methods for downstream Infinium analysis. In short, this treatment converts unmethylated cytosines to uracils while leaving methylated cytosines intact. DNA methylation was quantified at 863,904 CpG sites following hybridization to the Infinium MethylationEPIC BeadChip (Illumina) [32]. Beadchips were processed and scanned on the Illumina iScan at the University of Michigan (UM) Advanced Genomics Core using the Infinium Methylation EPIC kit.

We used a preprocessing pipeline for the resulting raw EPIC data based on a functional normalization method [33] within the 'minfi' package in R [34]. The pipeline included several steps: loading raw image files, linking to phenotypic information, dropping probes with SNPs within query sites, removing poor quality samples (>5% failed probes) and poorly detected probes (p-value>1e^{-6} when comparing to background), normalizing the data to correct for background signal and dye bias,

checking and correcting for batch effects (by slide and array), and removing CpG sites on the X and Y chromosomes. After QC and normalization, 786,024 CpG sites and 89 samples passed all QC measures and were subsequently used in statistical analysis.

Estimates of cell-type composition (T lymphocytes (CD4T, CD8T), B cells, NK cells, monocytes, granulocytes, and nucleated red blood cells) for each sample was performed using an established method based on UCB cell-type specific differentially methylation regions. Cell-type proportions were adjusted for in downstream statistical analysis to avoid confounding by cell-type composition [35]. Beta-values and M-values (log_2 ratio of the intensities of methylated probes versus unmethylated probes) were then calculated from the processed data and used in statistical analysis [36].

Statistical analysis

All statistical analyses were performed in the R Project for Statistical Computing (version 3.4.3). Analyses were performed to identify differentially methylated loci by Pb exposure. Pb biomarkers tested were maternal BLLs at each trimester, UCB Pb levels, and Pb levels in maternal patella and tibia, which were treated as non-transformed continuous variables. Bone and UCB Pb levels were normally distributed. Trimester-specific Pb measures were right-skewed yet all biomarker concentrations were biologically plausible and relevant to this study population. As such the relationships between maternal BLLs and DNA methylation were tested with and without BLL outliers. We performed bivariate analyses relating Pb biomarkers and covariates (e.g., cell-type proportions, gestational age, maternal age, maternal education, household income category) to identify potential confounders. We also performed singular value decomposition to identify covariates associated with principal components of the DNA

methylation data. We then chose potential confounding variables to include in the final statistical models of site-specific DNA methylation data that were associated with both Pb and DNA methylation. We also retained several variables that had a large effect on DNA methylation but were not also associated with Pb (i.e., infant sex, cell type proportions). In models with BLLs, offspring sex and estimated cell-type compositions of granulocytes and nucleated red blood cells (nRBC) [35] were included. Models with bone Pb measures as the predictor of interest additionally included maternal age and cohort. Other covariates available in this study population, including gestational age, were not considered as confounders because they were neither associated with prenatal Pb exposure nor DNA methylation in our study sample.

Differentially methylated positions (DMPs) by Pb were identified using the 'limma' package [37,38]. Briefly, linear regression models were run for each CpG site using M-values, which are the logit ratio of intensities of methylated probes versus unmethylated probes, with one Pb exposure biomarker as the predictor of interest and the aforementioned covariates included. Limma calculates fold-change in methylation, t-statistics, and p-values by applying empirical Bayes smoothing to the standard errors. To account for multiple testing, we used the false discovery rate (FDR) method [39], and consider DMPs with corrected p-value (q-value) <0.05 to be statistically significant. For comparison purposes between studies, we also report estimates (β) from the same regression analysis with betas (non-transformed methylation values) as the outcome.

We used the statistical package DMRcate to test associations between each Pb exposure variable and differential methylation at the regional level across the entire genome [40]. The Gaussian kernel bandwidth lambda values used was the default of

1000, and two CpG sites were considered the minimum consecutive loci to be included in a differentially methylated region (DMR). DMRs with FDR<0.05 were statistically significant.

We used LRpath, a multiple-comparison enrichment testing tool that uses logistic regression to test for gene sets that have higher significance values by raw p-value than expected at random [41], to identify gene pathways enriched for hyper- or hypo-methylation by Pb exposure. It considers CpG sites in known genes, their respective log fold changes by Pb from linear regression models, and associated p-values.

Results

Descriptive statistics of ELEMENT study data

Among the 89 mothers included in this analysis, 41 (46%) had male infants (**Table 2.1**). The mean age of women at offspring birth was 26.4 (SD=4.8) years. The average gestational age at birth was 39.0 (SD=1.1) weeks. The mean (SD) maternal BLLs averaged over all three trimesters was 6.18 (4.51) μg/dL, with the first trimester (T1) mean of 6.56 (5.35) μg/dL, second trimester (T2) mean of 5.93 (5.00) μg/dL, and third trimester (T3) mean of 6.09 (4.51) μg/dL. Maternal BLLs between the trimesters were highly correlated (r>0.63) according to a Spearman's rank-order correlation test. Average Pb concentration in patella was 9.42 (10.28) μg/g and tibia was 7.51 (9.46) μg/g. UCB Pb levels were measured to represent offspring Pb exposure at birth and averaged 4.86 (3.74) μg/dL. Geometric means of BLL among women in this study were more than four times higher than women of childbearing ages within a similar time period in the United States according to NHANES (1999-2002 and 2003-2006) [42].

Sources of Pb exposure in Mexico City during this time included the use of traditional lead-glazed ceramics for cooking, and Pb in gasoline which was not phased out in full until 1997 [43]. Pregnant women in countries such as Malaysia, India, Iran, Malta, and Bangladesh continue to have exposures similar or up to two-and-a-half times greater on average than that of the women in this study[44-48].

We compared participants included in this analysis with all ELEMENT mother-infant pairs from the same cohorts (2 and 3). Demographics and Pb biomarker concentrations were not statistically different between the subset and the entire study (**Table 2.1A Appendix**) with the exception of gestational age which was slightly higher in the subset with DNA methylation data.

CpG-site specific analysis and pathways analysis

We found that DNA methylation levels at three CpG sites demonstrated significant associations (FDR<0.05) with T1 BLLs, one CpG with T3 BLLs, and two with tibia bone Pb levels (**Table 2.2**). There were no statistically significant DMPs in analyses with maternal BLLs in T2, UCB Pb, or maternal patella bone Pb. Of the three statistically significant DMPs with the maternal BLLs during T1, the sites mapped to *RAB5A, EXT1,* and a non-genic region (chr8:12615485). The DMPs in *RAB5A* and *EXT1* had less DNA methylation (-0.22% and -0.02%, respectively) per 1-µg/dL increase in maternal T1 BLLs. In contrast, the non-genic region (chr8:12615485) was hypermethylated by Pb (0.35% per µg/dL of T1 BLL). The DMP by T3 maternal BLLs was hypomethylated (-0.52% per µg/dL BLL) and in a DNase I hypersensitivity site (chr1:160839299). Maternal tibia bone Pb associated DMPs were hypermethylated in

LNFN1 (0.37% per µg/g bone Pb) and hypomethylated in a non-genic region located at chr6:792305 (-0.21% per µg/g bone Pb).

LRpath analysis was conducted to test for gene sets enriched with differential methylation by each Pb biomarker. After multiple-comparisons correction, there were 109 gene sets enriched with T1 BLLs, 76 with T2 BLLs, 28 with T3 BLLs, and 12 with UCB Pb levels, as well as 15 with patella and 68 with tibia bone measures that met a q-value cut-off of 5% (**Table 2.2A Appendix**). Differently methylated genes associated with Pb exposure across all three trimesters and at birth were more likely to be enriched in gene pathways involved in detection of chemical stimulus, sensory perception and smell, immunoglobin binding, transmembrane receptor activity, and olfactory receptor activity. Four pathways were statistically significant (q-value<0.05) among all blood Pb variables, with 35 uniquely overlapping between T1 and T2, four uniquely overlapping between T2 and T3, none matching between T1 and T3 only, and two matching between T1 and at birth UCB (**Figure 2.1**). There were only four matching pathways when comparing results from models of tibia and patella bone Pb measures (**Figure 2.2A Appendix**).

Significant pathways from Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathways related to neurological development included neurological system processes associated with T1 (q-value =2.87E10^{-6}) and T2 (q-value=1.77E10^{-3}) BLLs and tibia bone Pb (q-value =5.65E10^{-9}). Nodal system pathway was also associated with T1 (q-value =1.40E10^{-3}) and T2 (q-value =1.36E10^{-3}) BLLs. Neurotransmitter receptor activity genes were associated with tibia bone Pb (q-value =7.84E10^{-3}).

Regional analysis

We tested for differentially methylated regions by Pb exposure for each trimester using DMRcate, which combines data from at least 2 consecutive CpG sites. DMRcate identified one region of differential methylation with T1 BLLs, one region with T3 BLLs, and one region with tibia bone Pb levels. No regions were identified with T2 BLLs, UCB Pb, or patella bone Pb. The region identified in T1 that was differentially methylated included 4 CpG sites in a region that is located between chr8:17433625-17433761 and is within the promotor of *PDGFRL* (**Figure 2.2**). The region identified in T3 included 12 CpG sites located at chr6:30095136-30095295 (**Figure 2.3**). Tibia bone Pb was associated with a region with 4 CpG sites within the promotor of *TRHR* (**Figure 2.4**). None of the DMPs identified before encompassed these regions.

Conclusion

We performed an epigenome-wide DNA methylation analysis using DNA extracted from nucleated UCB cells and the Illumina MethylationEPIC beadchip in 89 mother-infant pairs to identify differentially methylated genes by trimester-specific and cumulative Pb exposure biomarkers. To our knowledge, this is the first genome-wide DNA methylation study to investigate the association of DNA methylation at birth with prenatal Pb exposure at each of the three trimesters of pregnancy, as well as cumulative prenatal Pb exposure in maternal patella and tibia bones. We found that maternal BLLs during T1 and T3, as well as maternal tibia bone Pb levels, were associated with differential methylation at several CpG sites in UCB.

Epidemiological studies of Pb exposed pregnant women have reported BLLs below 5μg/dL can have adverse impacts on neurodevelopment, manifesting for example

as decreased scores in the neonatal behavioral neurological assessment test[49]. Maternal BLLs under 10μg/dL have been associated with a 3.5-6 point decrease in the Mental Development Index, a measure of IQ using the Bayley Scales of Infant Development, in 12-month-old infants (n=146) [29]. Although the inverse association between prenatal Pb exposure and neurodevelopment is known, whether epigenetic perturbation is one of the mechanisms underlying this association is not known.

In the epigenome-wide analysis performed here, the statistically significant DMPs and DMRs associated with biomarkers of prenatal Pb exposure annotated to several genes involved in neurodevelopment or neurologic function. In this study, DNA methylation levels at three CpG sites were significantly associated with maternal T1 BLLs, for which one is in a DHS within the first exon of *RAB5A*, a Ras-related protein. DHS are regulatory regions of the genome often located at transcription start sites where the DNA is accessible for transcription factor binding. *RAB5A* is an important mediator of endocytic pathways due to its role in intracellular membrane trafficking and fusion and localizing early endosomes [50-53]. Evidence from an *in vitro* study indicates that loss of *RAB5A* activity may perturb endosomal dynamics and may underlie neuronal dysfunction and degeneration in various motor neuron and neurodegenerative diseases [54]. The second CpG site associated with T1 BLLs is located at chr8:12615485, a DHS that is also classified to be within an enhancer region (according to FANTOM5 [55]) but unclassified to date in terms of the specific gene(s) it may regulate. The third CpG site is in a DHS, a suspected enhancer, and is within 200 base pairs of the transcription start site of *EXT1*, exostosin glycosyltransferase 1. *EXT1* has a primary role in the biosynthesis of heparan sulfate, which is known to regulate various biological

processes such as cell proliferation, growth factor signaling, and embryonic development, specifically mammalian neuronal development [56-59].

Maternal T1 Pb exposure was also associated with differential methylation in UCB at the regional level, identified by analyzing consecutive probes. We observed a region of 4 CpG sites located at chr8:17433625-17433761 within 200 base pairs of the transcription start site of *PDGFRL,* platelet derived growth factor receptor-like protein. This gene encodes a known tumor suppressor that inhibits the growth of colorectal cancer cells *in vitro*; the function of this gene in development, if any, is not yet known [60].

We observed one association between DNA methylation and T3 BLL. The CpG site associated with T3 BLLs is located at chr1:160839299 in an intergenic region within a DHS. In the regional analysis, there was one differentially methylated region that was statistically associated with maternal T3 Pb exposure. It annotated to chr6: 30095136-30095295 with 12 methylation sites within an intergenic region with no known regulatory function.

DNA methylation levels at two CpG sites were significantly associated with tibia bone Pb, a measure of cumulative Pb exposure throughout and before pregnancy. The first is within a DHS located in the gene *LRFN1*, Leucine Rich Repeat and Fibronectin Type III Domain Containing 1. LRFN proteins promote neurite outgrowth and synapse formation in hippocampal neurons and have a role in the developing nervous system with involvement in the regulation and conservation of excitatory synapses [61-63]. The second is located within a CpG island at chr6:792305 of no known regulatory function to date. We identified one region of differential methylation associated with tibia bone Pb located in the *TRHR* gene, thyrotropin-releasing hormone receptor, on chr8:110098835-

110098870 within 800 base pairs of the transcription start site. This receptor signals synthesis, secretion, and bioactivity of the thyroid stimulating hormone within the pituitary gland in the brain by activating the phosphatidylinositol-calcium-protein kinase C transduction pathway [64,65]. We did not identify associations between patella Pb and DNA methylation. Pb in tibia has a residence time of 25-30 years[66] while patella Pb has a half-life ranging from months to years[67]. While both serve as proxies for gestational Pb exposure, differences in half-life and bioavailability (i.e., following bone turnover) may influence the relationship between maternal bone Pb and fetal exposure dose throughout pregnancy.

We hypothesized that the greatest number of DMPs by Pb would be identified for T1, yet only a few significant results were obtained for T1, along with T3 and bone Pb. During early gestation, there are two major events influencing epigenetic programming: (1) post-fertilization there is a massive wave of demethylation to trigger embryonic developmental; (2) following, there is a subsequent re-methylation that shapes the epigenome in first cell lineages [68,69]. These events occur during early T1, and as such environmental perturbation during T1 could lead to epigenetic reprogramming that is propagated across all germ layers and tissues. Differentiation, which is critical for nervous system development, also occurs during T1 [70]. Throughout the remainder of fetal development, dividing somatic cells are methylated via maintenance epigenetic machinery [16]. Thus, while gestational exposures are expected to have the most dramatic effects on the DNA methylome across tissues, subtle changes can still occur in mid- to late-gestation that may be tissue-specific.

Our study is one of a growing body of literature that shows associations between gestational Pb exposure and offspring DNA methylation in rodent models and humans [19,20,71-75]. While the DMPs and DMRs identified in this study by T1 and T3 maternal BLL and maternal bone Pb were not the same as those previously reported in other cohorts, collectively these studies show that Pb exposure and DNA methylation vary by timing of exposure, sex, and population. A rural Bangladesh birth cohort study with high environmental Pb exposure performed epigenome-wide DNA methylation profiling using the Infinium HumanMethylation450K BeadChip (450K) on UCB and assessed its association with prenatal Pb exposure measured in maternal urine at 8 weeks' gestation and in maternal erythrocytes at 14 weeks gestation (n=127) [76]. This study identified nine loci associated with urine Pb and two loci with erythrocyte Pb. Another study, a U.S. prospective pregnancy cohort, Project Viva, with relatively low levels of Pb exposure (mean maternal erythrocyte Pb, 1.22±0.63μg/dL) conducted an epigenome-wide analysis using 450K on 268 UCB samples to evaluate the association between prenatal Pb exposure measured on average at 27.9 weeks' gestation and DNA methylation [20]. Their results identified four CpG sites in all newborns, as well as sex-specific CpG sites with more found in females (n=38) than males (n=2), associated with prenatal Pb. Differences in findings from these cohorts and ELEMENT could be due to differences in exposure levels between the cohorts, participant racial/ethnic background, sex differences, timing of Pb exposure assessment, and statistical power to detect true associations. Even so, our study adds to the previous evidence that prenatal Pb exposure is associated with altered DNA methylation with likely small effect sizes, and

this potential mechanism of toxicity or biomarker of response to exposure should be studied in larger cohorts and/or in consortiums.

While the mechanism by which Pb perturbs DNA methylation is not entirely known, several studies provide evidence for direct or indirect changes in DNMT expression and activity by Pb. DNMTs add methyl groups to CpG sites; if DMNTs fail to add methyl marks during cellular replication, DNA can become passively demethylated [77,78]. Pb exposure has been shown to increase reactive oxygen species which inhibits the DNMT binding to DNA causing hypomethylation at some but not all loci [21-23,79,80]. Additionally, several studies have provided evidence for Pb exposure effects on DMNT expression and function which could result in either increases or decreases in methylation [21,24-27].

Our study had several strengths including Pb exposure assessment at multiple time periods during gestation, epigenome-wide assessment of DNA methylation, a prospective study design, and rich data on key covariates. At the same time, our study has limitations. First, due to the sample size, we were unable to perform a sex-specific analysis to determine how sex may influence the relationship between DNA methylation and developmental Pb exposure. We were also underpowered to detect all 'true' differentially methylated genes by Pb exposure, especially those with small effect sizes. Secondly, although epigenomic changes in early gestation would be expected to propagate across all tissues, it is still important to consider tissue-specificity when conducting differential methylation studies. While brain is the primary tissue of interest in terms of Pb toxicity, blood-based epigenetic measures are typically necessary in longitudinal epidemiological studies. Cross-tissue studies should be conducted to better

understanding the biological connection between our findings in UCB with DNA methylation in the brain [81]. The Toxicant Exposures and Responses by Genomic and Epigenomic Regulators of Transcription (TaRGET II) consortium is currently promoting efforts towards understanding the role of environment in disease susceptibility as a function of epigenomic perturbations across target and surrogate tissue using mouse models (i.e. blood and brain), which will provide insights on cross-tissue, inter-interindividual variation of epigenomic marks [82]. Finally, although DNA methylation changes would be expected to alter gene expression, we are unable to test this hypothesis; storage conditions of archived UCB samples in this cohort did not allow for RNA isolation.

In conclusion, we found that T1, T3, and the cumulative measure in tibia of gestational Pb exposure were associated with several statistically-significant changes in DNA methylation. Five of the six CpG sites identified within the study were located within DHS which are important functional regions for gene regulation. There was no statistically significant differential methylation by T2 BLL, cord blood Pb, and patella bone Pb. Our findings are of importance because prenatal Pb exposure has been previously associated with adverse neurodevelopmental outcomes, but whether epigenetic mechanisms contribute to these long-term effects is not well characterized. Pb still remains a widespread environmental health problem. Dietary changes and micronutrient supplementation during pregnancy could play an essential role in neutralizing epigenomic perturbations due to environmental Pb exposure [83-85] , and the concentration of Pb in circulation can also be reduced by dietary supplementation (i.e. to calcium) [86]. Many public health promotion efforts are centered on primary prevention

of Pb exposure in early childhood even though gestation is also a critical developmental period when Pb can affect long-term health. Our results suggest that prenatal Pb exposure may modify DNA methylation profiles at birth, and this should be explored as one potential mechanism underlying Pb's neurodevelopmental effects.

Acknowledgements

I would like to thank my coauthors for their valuable input and feedback on this manuscript: Dana C Dolinoy, Wei Perng, Tamara R Jones, Marista Solano, Howard Hu, Mara M Tellez-Rojo, Karen E Peterson, and Jaclyn M Goodrich. The authors acknowledge the research staff at participating hospitals and the American British Cowdray Hospital in Mexico City for providing research facilities. We thank the mothers and children for participating in the study. This study was made possible by U.S. Environmental Protection Agency (US EPA) grants RD834800 and RD83543601 and National Institute for Environmental Health Sciences (NIEHS) grants P20 ES018171, P01 ES02284401, R01 ES007821, R01 ES014930, R01 ES013744, 1U2C ES026553, and P30 ES017885. This work was also supported by University of Michigan (UM) Genome Science Training Grant T32 HG000040 (CR). This study was also supported and partially funded by the National Institute of Public Health/Ministry of Health of Mexico.

Published

This chapter is a slightly modified version of a manuscript published in *Epigenetics Insights*:

Rygiel CA, Dolinoy DC, Perng W, Jones TR, Solano M, Hu H, Téllez-Rojo MM, Peterson KE, Goodrich JM. Trimester-Specific Associations of Prenatal Lead Exposure with Infant Cord Blood DNA Methylation at Birth. *Epigenetic Insights.* 2020 Jul 20;13:2516865720938669. doi: 10.1177/2516865720938669. PMID: 32734142; PMCID: PMC7372614.

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Tables and Figures

Table 2.1: Characteristics of ELEMENT mother-infant pairs with UCB DNA methylation data.

Characteristics	No.	Mean ± SD or N (%)	Range
Mothers			
Age (yrs.)	89	26.4 ± 4.81	18.0 - 37.0
Blood lead (µg/dL)			
First Trimester (T1)	69	6.56 ± 5.35	0.90 - 35.8
Second Trimester (T2)	74	5.93 ± 5.00	0.80 - 38.2
Third Trimester (T3)	76	6.09 ± 4.51	0.90 - 34.0
Average All Trimesters	77	6.18 ± 4.51	1.17 - 33.1
Bone lead (µg/g)			
Patella	73	11.8 ± 9.25	0.20 - 42.0
Tibia	46	11.8 ± 6.73	0.40 - 28.8
Household Income	77		
Lowest		12 (15.6)	
Low-Middle		31 (40.3)	
Middle		20 (26.0)	
Middle-High		9 (11.6)	
Highest		5 (6.5)	
Maternal education (total yrs.)	89	11.0 ± 2.39	3.00 - 17.0
Children			
UCB lead (µg/dL)	86	4.86 ± 3.74	0.00 - 19.5
Gestational age (wks.)	87	39.0 ± 1.09	36.0 - 41.0
Male (%)	89	41 (46.0)	

Note: SD, standard deviation; UCB, umbilical cord blood

Table 2.2: Statistically significant DMPs (q<0.05) by first trimester maternal BLL, third trimester maternal BLL, and maternal tibia Pb.

Pb Biomarker	Probe ID	Gene Name	Chr	Pos	β estimate	P-Value	Q-Value
T1 BLL	cg17138393	*RAB5A*	chr3	19988887	-0.000221	5.46E-08	0.0227
	cg03390844		chr8	12615485	0.00348	5.78E-08	0.0227
	cg00984923	*EXT1*	chr8	119124069	-0.000235	1.49E-07	0.0390
T3 BLL	cg01328348		chr1	160839299	-0.00519	1.81E-08	0.0142
Tibia Pb	cg00002033	*LRFN1*	chr19	39798481	0.00367	3.57E-08	0.0281
	cg03463208		chr6	792305	-0.00205	7.23E-08	0.0284

Note: Results from the analysis are shown for CpG sites associated with Pb biomarker levels below q-value of 0.05 from models adjusted for sex and estimated cell-type proportions (granulocytes and nucleated red blood cells). The p-values and q-values are obtained from the analyses using methylation expressed as M-values as the outcome variable and reported association estimates (β) are from analysis using methylation beta values as the outcome variable. chr, chromosome; pos, base pair position from hg19.

Figure 2.1: Venn diagram of functional annotations (q-value<0.05) associated with maternal Pb exposure compared across all three trimesters (first trimester, T1; second trimester, T2; third trimester, T3) and umbilical cord blood (UCB).

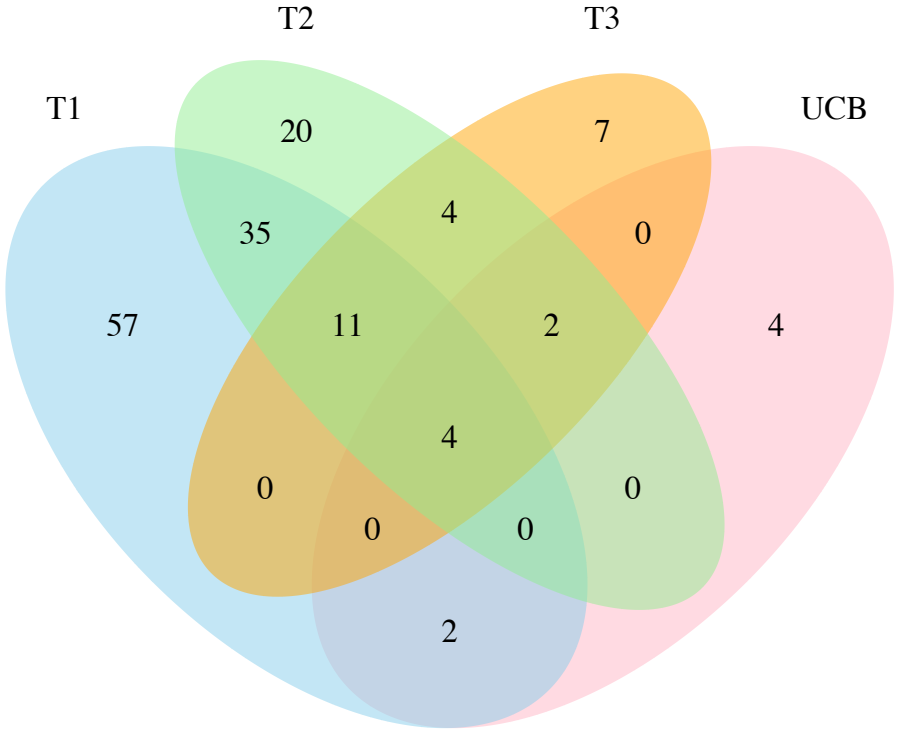


Figure 2.2: Differentially methylated region (DMR) by maternal blood Pb concentrations in T1 located within 200 base pairs of the transcription start site of PDGFRL (chr8:17433625-17433761). Linear regression modeling, treating Pb exposure as a continuous variable, adjusted for sex and estimated cell-type proportions. DNA methylation (beta value) is plotted for each sample at six CpG sites in the region included four sites of the statistically significant DMR. Exposure quartile ranges are represented from light red to dark red with quartile 4 (Q4), representing the top 25% most Pb exposed during T1, being the most methylated at each of the four CpG sites within the DMR.

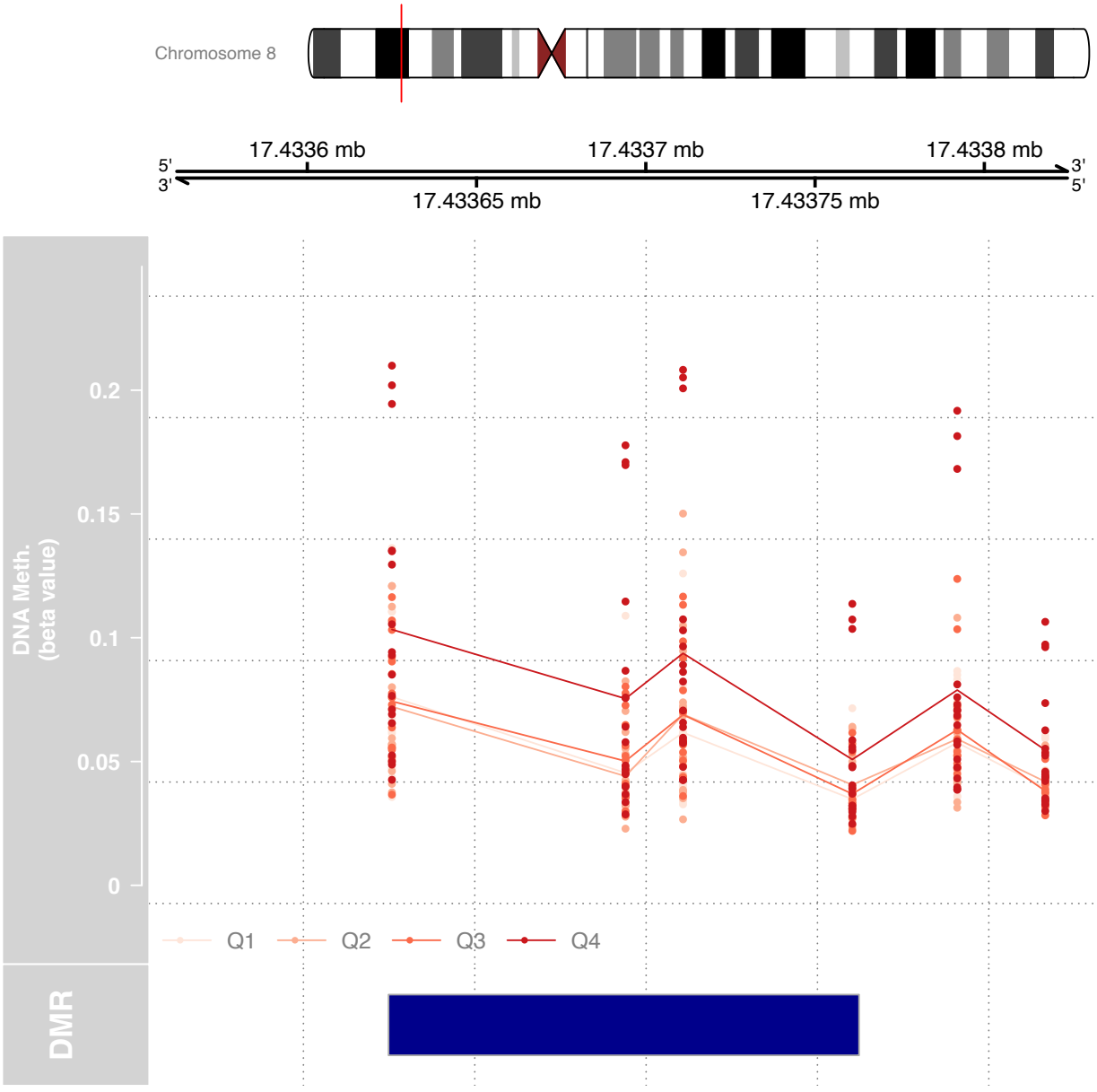


Figure 2.3: DMR by maternal blood Pb concentrations in T3 at chr6:30095136-30095295 includes 12 CpG sites within an intergenic region with no known regulatory function. This DMR was selected from a linear regression modeling, treating Pb exposure as a continuous variable, adjusting for sex and estimated cell-type proportions. DNA methylation (beta value) is plotted for each sample at the twelve CpG sites in the DMR. Exposure quartile ranges are represented from light red to dark red with Q4, representing the top 25% most Pb exposed during T3, being the most methylated at each of the twelve CpG sites within the DMR.

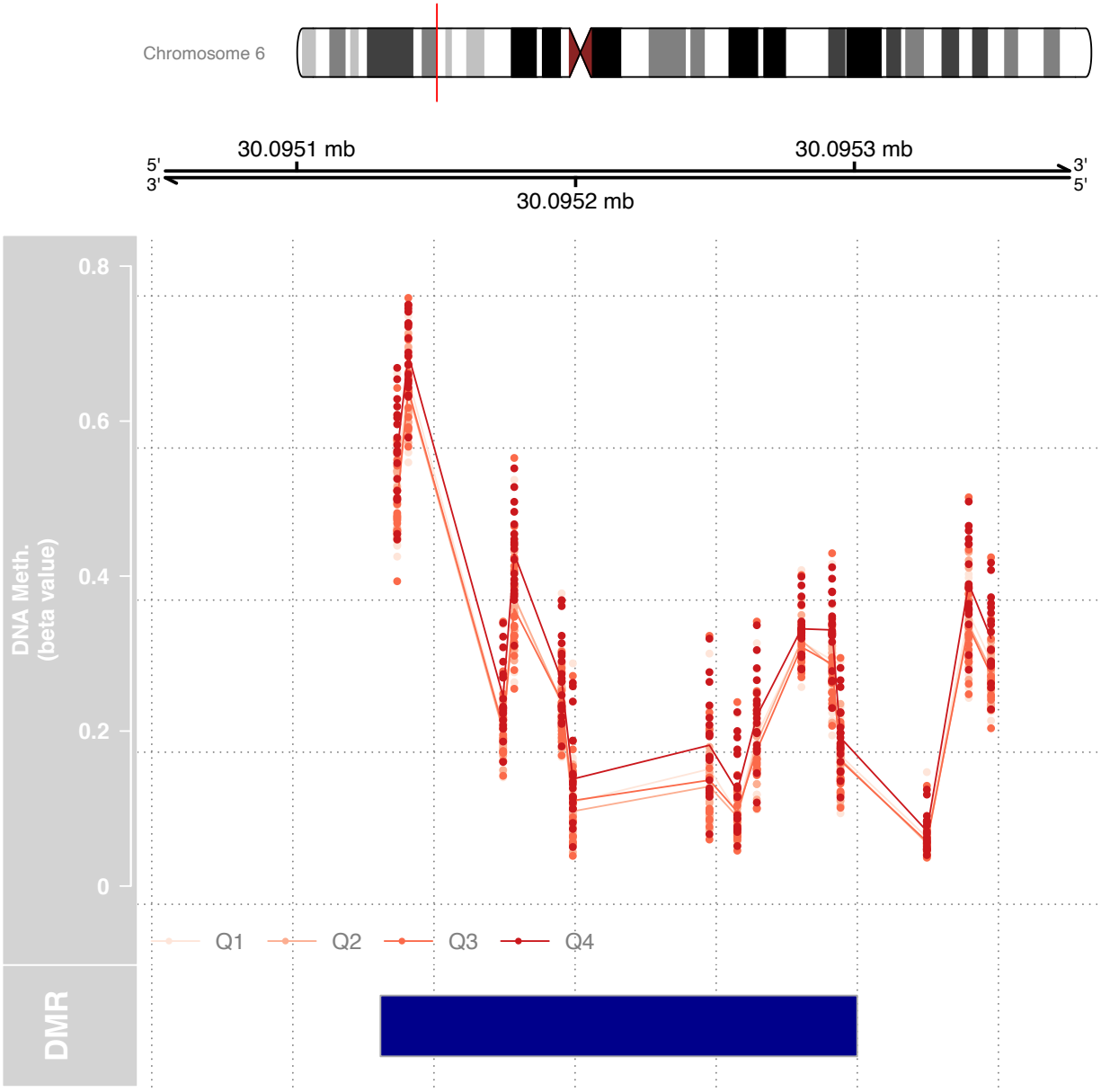
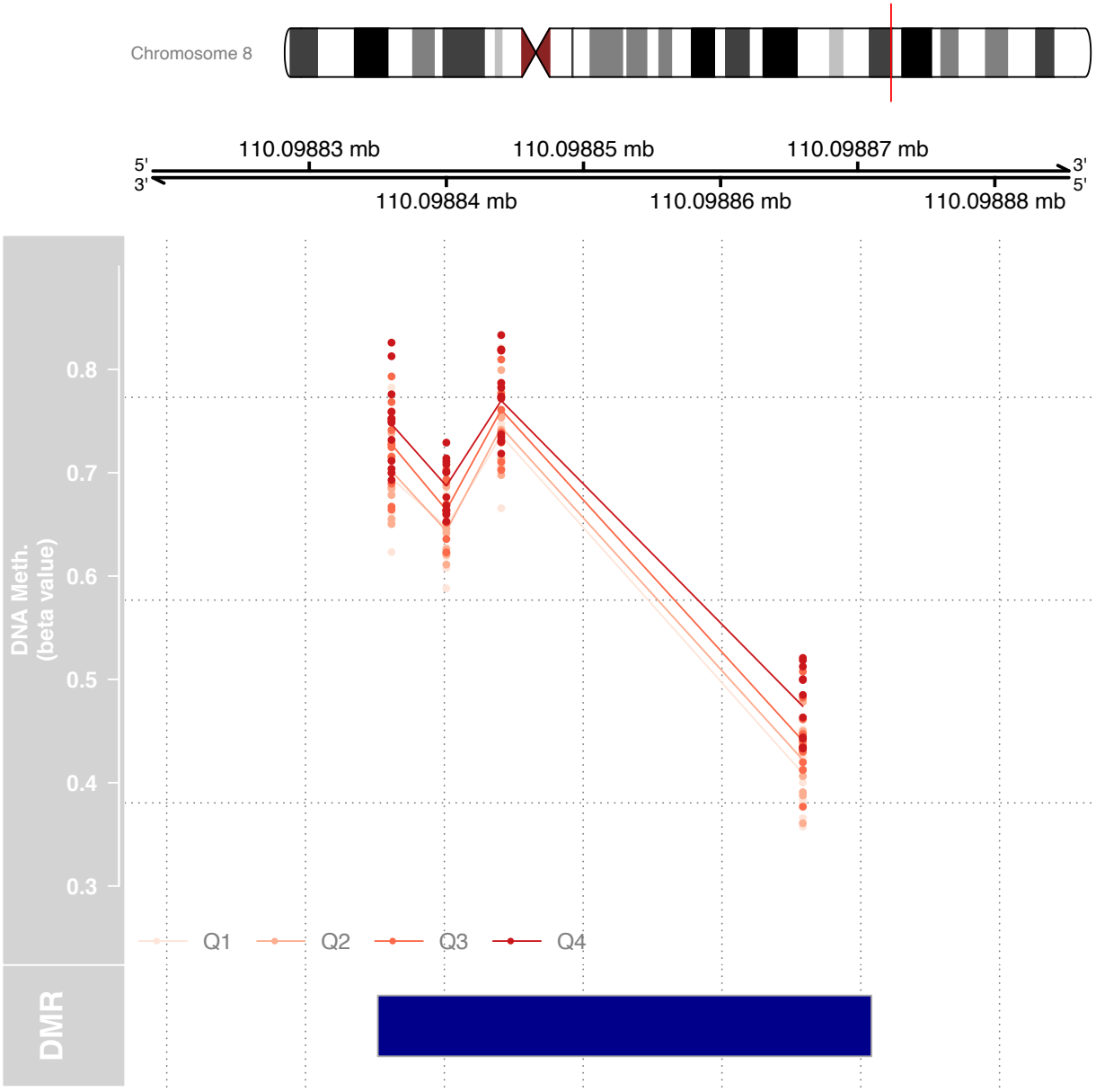


Figure 2.4: DMR by maternal tibia bone Pb located within 800 base pairs of the transcription start site of TRHR (chr8:110098835-110098870). The DMR was identified by a linear regression model, treating Pb exposure as a continuous variable, and adjusting for sex and estimated cell-type proportions, maternal age, and cohort. DNA methylation (beta value) is plotted for each sample for the four CpG sites in the DMR. Exposure quartile ranges are represented from light red to dark red with Q4, representing the top 25% most Pb exposed when cumulative Pb is measured in tibia, being the most methylated at each of the four CpG sites within the DMR.



Appendix

Table 2.1A: Comparison of all ELEMENT cohort 2 and 3 participants with subset of ELEMENT included in the current study.

	ELEMENT Subset**		All ELEMENT**		
Characteristics	**No.**	**Mean ± SD or N (%)**	**No.**	**Mean ± SD or N (%)**	**P-Value**
Mothers					
Age (yrs.)	89	26.4 ± 4.81	1213	26.1 ± 5.34	0.34
Blood lead (µg/dL)					
First Trimester (T1)	69	6.56 ± 5.35	594	5.77 ± 4.03	0.19
Second Trimester (T2)	74	5.93 ± 5.00	616	5.20 ± 3.93	0.11
Third Trimester (T3)	76	6.09 ± 4.51	575	5.54 ± 4.13	0.12
Average All Trimesters	77	6.18 ± 4.51	643	5.53 ± 3.51	0.13
Bone lead (µg/g)					
Patella	72	11.8 ± 9.25	894	12.3 ± 8.69	0.41
Tibia	46	11.8 ± 6.73	666	11.4 ± 7.70	0.46
Household Income	77		688		0.91
Lowest		12 (15.6)		117 (17.0)	
Low-Middle		31 (40.3)		251 (36.5)	
Middle		20 (26.0)		213 (31.0)	
Middle-High		9 (11.6)		82 (11.9)	
Highest		5 (6.5)		25 (3.6)	
Education (total yrs.)	89	11.0 ± 2.39	1212	10.8 ± 2.99	0.22
Children					
UCB lead (µg/dL)	86	4.86 ± 3.74	462	4.45 ± 3.00	0.74
Gestational age (wks.)	87	39.0 ± 1.09	1196	38.8 ± 1.57	0.03*
Male (%)	89	41 (46.0)	1202	621 (51.7)	0.36

Note: SD, standard deviation; UCB, umbilical cord blood

* p-value < 0.05 using Wilcox signed-rank test

** Participants for the current study were a subset of mother-child pairs from Cohorts 2 and 3 of ELEMENT that had archived cord blood samples for DNA methylation analysis. Here we are comparing their characteristics to those of all ELEMENT women and their newborns from the same cohorts (cohorts 2 and 3) that had at least one Pb biomarker available (n=1214).

Table 2.2A: Statistically significantly enriched functional annotations using LRpath (q-value<0.05) from epigenome-wide analysis of DNA methylation and biomarkers of Pb.

Biomarker	Name	Database	Direction	Coeff.	P-Value	Q-Value
T1	detection of chemical stimulus involved in sensory perception	GOBP	up	1.31	1.8E-21	1.1E-17
	detection of chemical stimulus	GOBP	up	1.28	6.6E-21	2.0E-17
	sensory perception of chemical stimulus	GOBP	up	1.23	2.1E-19	4.0E-16
	detection of stimulus involved in sensory perception	GOBP	up	1.23	2.6E-19	4.0E-16
	odorant binding	GOMF	up	1.59	4.0E-18	4.5E-15
	detection of chemical stimulus involved in sensory perception of smell	GOBP	up	1.23	1.7E-17	2.1E-14
	olfactory receptor activity	GOMF	up	1.20	5.0E-17	2.8E-14
	detection of stimulus	GOBP	up	1.10	3.2E-17	3.3E-14
	sensory perception of smell	GOBP	up	1.18	1.6E-16	1.4E-13
	intracellular	GOCC	down	-0.69	7.0E-15	2.7E-12
	organelle	GOCC	down	-0.68	3.8E-15	2.7E-12
	intracellular part	GOCC	down	-0.68	1.2E-14	3.0E-12
	membrane-bounded organelle	GOCC	down	-0.62	2.8E-13	5.4E-11
	intracellular organelle	GOCC	down	-0.61	7.3E-13	1.1E-10
	sensory perception	GOBP	up	0.91	2.4E-13	1.8E-10
	Olfactory transduction	KEGG	up	1.30	1.0E-12	2.2E-10
	intracellular membrane-bounded organelle	GOCC	down	-0.58	9.8E-12	1.3E-09
	G-protein coupled receptor activity	GOMF	up	0.86	1.7E-11	6.3E-09
	type I interferon receptor binding	GOMF	up	1.74	3.1E-10	7.0E-08
	IgG binding	GOMF	up	1.79	3.0E-10	7.0E-08
	bitter taste receptor activity	GOMF	up	1.63	4.3E-10	8.2E-08
	cytoplasm	GOCC	down	-0.50	1.5E-09	1.7E-07
	transmembrane receptor activity	GOMF	up	0.71	1.6E-09	2.6E-07
	transmembrane signaling receptor activity	GOMF	up	0.71	2.2E-09	3.1E-07
	positive regulation of peptidyl-serine phosphorylation of STAT protein	GOBP	up	1.71	6.5E-10	4.4E-07
	taste receptor activity	GOMF	up	1.52	3.7E-09	4.7E-07
	regulation of peptidyl-serine phosphorylation of STAT protein	GOBP	up	1.68	1.0E-09	6.2E-07
	serine phosphorylation of STAT protein	GOBP	up	1.61	1.9E-09	1.1E-06
	G-protein coupled receptor signaling pathway	GOBP	up	0.72	2.2E-09	1.1E-06
	organelle part	GOCC	down	-0.49	1.7E-08	1.7E-06
	signaling receptor activity	GOMF	up	0.66	2.1E-08	2.4E-06
	neurological system process	GOBP	up	0.70	6.1E-09	2.9E-06
	cytoplasmic part	GOCC	down	-0.48	3.5E-08	2.9E-06
	intracellular organelle part	GOCC	down	-0.47	4.3E-08	3.3E-06
	receptor activity	GOMF	up	0.62	3.5E-08	3.3E-06
	molecular transducer activity	GOMF	up	0.62	3.5E-08	3.3E-06
	immunoglobulin binding	GOMF	up	1.51	5.6E-08	4.9E-06

	detection of chemical stimulus involved in sensory perception of bitter taste	GOBP	up	1.44	2.8E-08	1.2E-05
	cell	GOCC	down	-0.55	1.9E-07	1.3E-05
	cell part	GOCC	down	-0.54	2.5E-07	1.6E-05
	natural killer cell activation involved in immune response	GOBP	up	1.51	4.0E-08	1.6E-05
	detection of chemical stimulus involved in sensory perception of taste	GOBP	up	1.37	1.1E-07	4.1E-05
	signal transducer activity	GOMF	up	0.57	6.0E-07	4.9E-05
	nucleus	GOCC	down	-0.44	1.0E-06	6.1E-05
	sensory perception of bitter taste	GOBP	up	1.35	2.6E-07	9.3E-05
	endonuclease activity, active with either ribo- or deoxyribonucleic acids and producing 3'-phosphomonoesters	GOMF	up	1.41	4.4E-06	3.4E-04
	cellular component organization or biogenesis	GOBP	down	-0.47	1.8E-06	6.1E-04
	primary metabolic process	GOBP	down	-0.42	1.9E-06	6.1E-04
	endodeoxyribonuclease activity, producing 5'-phosphomonoesters	GOMF	up	1.54	8.7E-06	6.1E-04
	organic substance metabolic process	GOBP	down	-0.42	2.2E-06	6.7E-04
	Regulation of autophagy	KEGG	up	1.53	6.7E-06	7.1E-04
	cellular component organization	GOBP	down	-0.46	3.8E-06	1.1E-03
	response to exogenous dsRNA	GOBP	up	1.28	4.2E-06	1.2E-03
	metabolic process	GOBP	down	-0.40	5.2E-06	1.4E-03
	nodal signaling pathway	GOBP	up	1.54	5.5E-06	1.4E-03
	organic cyclic compound binding	GOMF	down	-0.42	2.3E-05	1.5E-03
	humoral immune response	GOBP	up	0.95	7.3E-06	1.8E-03
	cellular metabolic process	GOBP	down	-0.39	9.2E-06	2.1E-03
	sensory perception of taste	GOBP	up	1.15	9.7E-06	2.1E-03
	localization	GOBP	down	-0.44	9.6E-06	2.1E-03
	protein binding	GOMF	down	-0.37	3.8E-05	2.3E-03
	endodeoxyribonuclease activity, producing 3'-phosphomonoesters	GOMF	up	1.49	3.9E-05	2.3E-03
	Taste transduction	KEGG	up	1.31	3.5E-05	2.5E-03
	heterocyclic compound binding	GOMF	down	-0.40	4.7E-05	2.7E-03
	macromolecular complex	GOCC	down	-0.40	6.2E-05	3.4E-03
	oxygen binding	GOMF	up	1.14	6.6E-05	3.5E-03
	RIG-I-like receptor signaling pathway	KEGG	up	1.21	7.9E-05	3.9E-03
	Staphylococcus aureus infection	KEGG	up	1.26	9.1E-05	3.9E-03
	nitrogen compound metabolic process	GOBP	down	-0.40	1.8E-05	3.9E-03
	negative regulation of lipopolysaccharide-mediated signaling pathway	GOBP	up	1.58	2.1E-05	4.2E-03
	defense response to bacterium	GOBP	up	0.86	2.1E-05	4.2E-03
	protein complex	GOCC	down	-0.42	9.3E-05	4.7E-03
	cytokine activity	GOMF	up	0.80	9.7E-05	5.0E-03
	macromolecule localization	GOBP	down	-0.56	3.3E-05	6.3E-03
	pattern binding	GOMF	up	1.31	1.8E-04	8.5E-03
	polysaccharide binding	GOMF	up	1.31	1.8E-04	8.5E-03

	macromolecule metabolic process	GOBP	down	-0.36	5.2E-05	9.6E-03
	phosphatidylcholine binding	GOMF	down	-2.48	2.2E-04	9.8E-03
	B cell proliferation	GOBP	up	1.04	6.2E-05	1.1E-02
	cellular macromolecule metabolic process	GOBP	down	-0.35	7.4E-05	1.3E-02
	sperm principal piece	GOCC	down	-2.59	2.7E-04	1.3E-02
	heterocycle metabolic process	GOBP	down	-0.39	8.3E-05	1.4E-02
	regulation of type I interferon-mediated signaling pathway	GOBP	up	1.21	8.6E-05	1.4E-02
	regulation of lipopolysaccharide-mediated signaling pathway	GOBP	up	1.37	9.1E-05	1.5E-02
	organic substance biosynthetic process	GOBP	down	-0.38	9.3E-05	1.5E-02
	cytosol	GOCC	down	-0.41	3.3E-04	1.5E-02
	cellular aromatic compound metabolic process	GOBP	down	-0.38	1.1E-04	1.6E-02
	cellular nitrogen compound metabolic process	GOBP	down	-0.37	1.0E-04	1.6E-02
	cellular biosynthetic process	GOBP	down	-0.37	1.2E-04	1.6E-02
	negative regulation of serine-type endopeptidase activity	GOBP	up	1.52	1.2E-04	1.6E-02
	negative regulation of serine-type peptidase activity	GOBP	up	1.52	1.2E-04	1.6E-02
	Drug metabolism - cytochrome P450	KEGG	up	1.10	4.7E-04	1.7E-02
	nuclear part	GOCC	down	-0.38	4.0E-04	1.7E-02
	biosynthetic process	GOBP	down	-0.36	1.4E-04	1.9E-02
	T cell activation involved in immune response	GOBP	up	1.00	1.7E-04	2.2E-02
	extracellular space	GOCC	up	0.41	5.5E-04	2.2E-02
	organic cyclic compound metabolic process	GOBP	down	-0.36	2.0E-04	2.6E-02
	nucleic acid binding	GOMF	down	-0.38	6.2E-04	2.7E-02
	complement receptor activity	GOMF	down	-2.68	6.8E-04	2.8E-02
	protein localization	GOBP	down	-0.52	2.4E-04	3.1E-02
	lipopolysaccharide binding	GOMF	up	1.23	8.4E-04	3.4E-02
	catalytic activity	GOMF	down	-0.32	9.3E-04	3.6E-02
	positive regulation of peptidyl-serine phosphorylation	GOBP	up	0.98	3.1E-04	3.8E-02
	defense response to other organism	GOBP	up	0.63	3.3E-04	4.1E-02
	regulation of gene expression	GOBP	down	-0.39	4.0E-04	4.7E-02
	cellular macromolecule biosynthetic process	GOBP	down	-0.37	3.9E-04	4.7E-02
	nucleobase-containing compound metabolic process	GOBP	down	-0.34	4.3E-04	4.9E-02
	regulation of cellular macromolecule biosynthetic process	GOBP	down	-0.40	4.3E-04	4.9E-02
	Drug metabolism - other enzymes	KEGG	up	1.13	1.6E-03	5.0E-02
T2	olfactory receptor activity	GOMF	up	1.21	3.1E-17	3.6E-14
	detection of chemical stimulus involved in sensory perception	GOBP	up	1.15	5.2E-17	1.9E-13
	detection of chemical stimulus involved in sensory perception of smell	GOBP	up	1.17	6.1E-17	1.9E-13
	sensory perception of chemical stimulus	GOBP	up	1.11	1.3E-16	2.0E-13
	sensory perception of smell	GOBP	up	1.14	1.3E-16	2.0E-13
	detection of chemical stimulus	GOBP	up	1.11	2.8E-16	3.5E-13
	detection of stimulus involved in sensory perception	GOBP	up	1.10	5.7E-16	5.8E-13
	odorant binding	GOMF	up	1.47	5.0E-15	2.8E-12

	intracellular	GOCC	down	-0.65	1.2E-12	9.5E-10
	intracellular part	GOCC	down	-0.63	2.8E-12	1.1E-09
	Olfactory transduction	KEGG	up	1.30	7.2E-12	1.5E-09
	detection of stimulus	GOBP	up	0.93	1.8E-12	1.5E-09
	organelle	GOCC	down	-0.61	8.7E-12	2.2E-09
	IgG binding	GOMF	up	1.89	5.9E-12	2.2E-09
	G-protein coupled receptor activity	GOMF	up	0.88	2.0E-11	5.7E-09
	intracellular organelle	GOCC	down	-0.55	2.8E-10	5.4E-08
	immunoglobulin binding	GOMF	up	1.62	8.6E-10	2.0E-07
	cytoplasm	GOCC	down	-0.52	3.3E-09	5.1E-07
	sensory perception	GOBP	up	0.78	9.2E-10	7.0E-07
	membrane-bounded organelle	GOCC	down	-0.51	6.0E-09	7.6E-07
	intracellular membrane-bounded organelle	GOCC	down	-0.46	8.6E-08	9.4E-06
	transmembrane receptor activity	GOMF	up	0.65	1.6E-07	3.0E-05
	transmembrane signaling receptor activity	GOMF	up	0.65	2.5E-07	4.1E-05
	Systemic lupus erythematosus	KEGG	up	1.15	5.7E-07	6.1E-05
	organelle part	GOCC	down	-0.42	1.5E-06	1.4E-04
	type I interferon receptor binding	GOMF	up	1.56	1.3E-06	1.8E-04
	signaling receptor activity	GOMF	up	0.60	1.4E-06	1.8E-04
	intracellular organelle part	GOCC	down	-0.42	2.6E-06	2.2E-04
	G-protein coupled receptor signaling pathway	GOBP	up	0.63	3.5E-07	2.4E-04
	cytoplasmic part	GOCC	down	-0.41	3.4E-06	2.6E-04
	receptor activity	GOMF	up	0.56	3.2E-06	3.3E-04
	molecular transducer activity	GOMF	up	0.56	3.2E-06	3.3E-04
	cell	GOCC	down	-0.49	1.0E-05	7.1E-04
	protein binding	GOMF	down	-0.41	1.0E-05	9.7E-04
	cell part	GOCC	down	-0.47	1.7E-05	1.1E-03
	complement binding	GOMF	up	1.45	1.5E-05	1.3E-03
	nodal signaling pathway	GOBP	up	1.51	2.2E-06	1.4E-03
	neurological system process	GOBP	up	0.58	3.2E-06	1.8E-03
	Staphylococcus aureus infection	KEGG	up	1.17	4.0E-05	2.8E-03
	opsonin binding	GOMF	up	1.55	3.7E-05	3.0E-03
	signal transducer activity	GOMF	up	0.49	3.9E-05	3.0E-03
	Antigen processing and presentation	KEGG	down	-1.74	7.2E-05	3.8E-03
	humoral immune response	GOBP	up	0.92	7.6E-06	3.9E-03
	regulation of humoral immune response	GOBP	up	1.17	8.9E-06	4.2E-03
	positive regulation of peptidyl-serine phosphorylation of STAT protein	GOBP	up	1.41	9.5E-06	4.2E-03
	localization	GOBP	down	-0.45	1.4E-05	5.6E-03
	regulation of peptidyl-serine phosphorylation of STAT protein	GOBP	up	1.38	1.8E-05	7.0E-03
	negative regulation of heart contraction	GOBP	down	-2.59	3.0E-05	1.1E-02
	cation-transporting ATPase complex	GOCC	down	-2.42	2.0E-04	1.2E-02

	natural killer cell activation involved in immune response	GOBP	up	1.28	3.7E-05	1.2E-02
	single-multicellular organism process	GOBP	down	-0.42	3.6E-05	1.2E-02
	2 iron, 2 sulfur cluster binding	GOMF	up	1.29	2.1E-04	1.5E-02
	epithelial cilium movement	GOBP	down	-2.56	6.5E-05	2.0E-02
	serine phosphorylation of STAT protein	GOBP	up	1.28	8.7E-05	2.5E-02
	cellular component organization or biogenesis	GOBP	down	-0.39	9.9E-05	2.7E-02
	defense response to bacterium	GOBP	up	0.79	1.1E-04	2.8E-02
	regulation of complement activation	GOBP	up	1.21	1.2E-04	2.9E-02
	blood microparticle	GOCC	up	0.84	6.2E-04	3.4E-02
	WASH complex	GOCC	up	1.35	7.1E-04	3.6E-02
	beta-amyloid metabolic process	GOBP	down	-2.40	1.5E-04	3.7E-02
	iron-sulfur cluster assembly	GOBP	up	1.33	1.7E-04	3.9E-02
	metallo-sulfur cluster assembly	GOBP	up	1.33	1.7E-04	3.9E-02
	multicellular organism development	GOBP	down	-0.41	1.9E-04	4.0E-02
	negative regulation of blood circulation	GOBP	down	-2.25	2.0E-04	4.0E-02
	regulation of protein activation cascade	GOBP	up	1.18	1.9E-04	4.0E-02
	intramolecular oxidoreductase activity, transposing C=C bonds	GOMF	up	1.44	6.0E-04	4.0E-02
	release of sequestered calcium ion into cytosol by sarcoplasmic reticulum	GOBP	down	-2.29	2.5E-04	4.3E-02
	cellular component organization	GOBP	down	-0.36	2.7E-04	4.3E-02
	macromolecule localization	GOBP	down	-0.50	2.6E-04	4.3E-02
	anatomical structure development	GOBP	down	-0.38	2.3E-04	4.3E-02
	organic substance transport	GOBP	down	-0.52	2.4E-04	4.3E-02
	defense response to other organism	GOBP	up	0.63	2.5E-04	4.3E-02
	negative regulation of calcium ion transmembrane transporter activity	GOBP	down	-2.37	2.7E-04	4.3E-02
	calcium ion transport from endoplasmic reticulum to cytosol	GOBP	down	-2.29	2.5E-04	4.3E-02
	ferrous iron binding	GOMF	up	1.26	7.0E-04	4.4E-02
	Regulation of autophagy	KEGG	up	1.14	1.2E-03	5.0E-02
T3	olfactory receptor activity	GOMF	up	0.97	4.1E-09	4.5E-06
	IgG binding	GOMF	up	1.74	8.0E-09	4.5E-06
	immunoglobulin binding	GOMF	up	1.50	5.1E-08	1.9E-05
	detection of chemical stimulus involved in sensory perception of smell	GOBP	up	0.91	7.5E-09	4.6E-05
	sensory perception of smell	GOBP	up	0.87	3.3E-08	1.0E-04
	Olfactory transduction	KEGG	up	0.95	1.4E-06	2.9E-04
	detection of chemical stimulus involved in sensory perception	GOBP	up	0.81	2.3E-07	4.6E-04
	sensory perception of chemical stimulus	GOBP	up	0.77	5.9E-07	7.9E-04
	detection of chemical stimulus	GOBP	up	0.78	6.5E-07	7.9E-04
	G-protein coupled receptor activity	GOMF	up	0.67	3.7E-06	1.0E-03
	Systemic lupus erythematosus	KEGG	up	1.06	1.0E-05	1.1E-03
	detection of stimulus involved in sensory perception	GOBP	up	0.76	1.2E-06	1.2E-03
	ferrous iron binding	GOMF	up	1.29	1.2E-05	2.4E-03
	2 iron, 2 sulfur cluster binding	GOMF	up	1.28	1.2E-05	2.4E-03

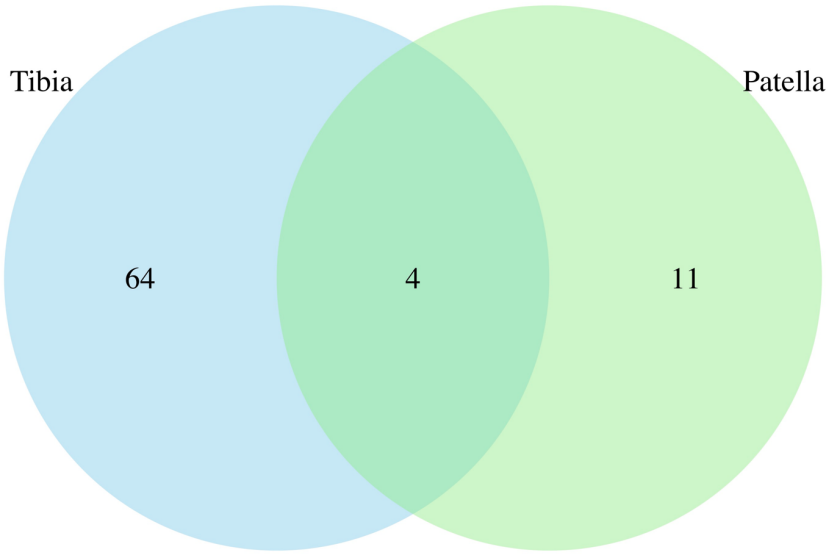
	iron-sulfur cluster assembly	GOBP	up	1.29	3.9E-06	3.0E-03
	metallo-sulfur cluster assembly	GOBP	up	1.29	3.9E-06	3.0E-03
	WASH complex	GOCC	up	1.36	4.0E-06	3.1E-03
	G-protein coupled nucleotide receptor activity	GOMF	up	1.34	9.8E-05	1.4E-02
	G-protein coupled purinergic nucleotide receptor activity	GOMF	up	1.34	9.8E-05	1.4E-02
	transmembrane signaling receptor activity	GOMF	up	0.50	2.0E-04	2.5E-02
	transmembrane receptor activity	GOMF	up	0.49	2.2E-04	2.5E-02
	sarcoplasmic reticulum calcium ion transport	GOBP	down	-1.99	3.7E-05	2.5E-02
	odorant binding	GOMF	up	0.93	2.8E-04	2.7E-02
	4 iron, 4 sulfur cluster binding	GOMF	up	1.06	2.9E-04	2.7E-02
	cellular defense response	GOBP	down	-1.79	5.0E-05	3.0E-02
	signaling receptor activity	GOMF	up	0.46	5.0E-04	4.3E-02
	purinergic nucleotide receptor activity	GOMF	up	1.17	6.0E-04	4.5E-02
	nucleotide receptor activity	GOMF	up	1.17	6.0E-04	4.5E-02
UCB Pb	IgG binding	GOMF	up	1.55	2.0E-08	2.3E-05
	immunoglobulin binding	GOMF	up	1.37	3.6E-07	2.0E-04
	endodeoxyribonuclease activity, producing 5'-phosphomonoesters	GOMF	down	-2.61	8.7E-06	3.3E-03
	olfactory receptor activity	GOMF	up	0.76	1.5E-05	4.3E-03
	2 iron, 2 sulfur cluster binding	GOMF	up	1.20	7.7E-05	1.7E-02
	Chemokine signaling pathway	KEGG	down	-1.26	1.0E-04	1.8E-02
	Olfactory transduction	KEGG	up	0.79	1.7E-04	1.8E-02
	Ribosome	KEGG	up	1.04	3.1E-04	2.2E-02
	chemokine activity	GOMF	down	-1.83	1.6E-04	3.0E-02
	endodeoxyribonuclease activity, producing 3'-phosphomonoesters	GOMF	down	-2.42	2.3E-04	3.8E-02
	ferrous iron binding	GOMF	up	1.18	2.7E-04	3.9E-02
	transcriptional activator activity, RNA polymerase II distal enhancer sequence-specific binding	GOMF	up	1.15	3.2E-04	4.0E-02
Tibia	olfactory receptor activity	GOMF	down	-2.11	2.8E-29	3.1E-26
	sensory perception of smell	GOBP	down	-1.90	3.2E-24	6.5E-21
	detection of chemical stimulus involved in sensory perception	GOBP	down	-1.89	2.3E-24	6.5E-21
	detection of chemical stimulus involved in sensory perception of smell	GOBP	down	-1.94	1.1E-24	6.5E-21
	Olfactory transduction	KEGG	down	-2.57	2.0E-22	4.2E-20
	sensory perception of chemical stimulus	GOBP	down	-1.80	3.2E-23	4.9E-20
	detection of chemical stimulus	GOBP	down	-1.80	5.6E-23	6.8E-20
	detection of stimulus involved in sensory perception	GOBP	down	-1.78	1.3E-22	1.3E-19
	G-protein coupled receptor activity	GOMF	down	-1.45	1.3E-19	7.4E-17
	detection of stimulus	GOBP	down	-1.51	9.7E-19	8.4E-16
	sensory perception	GOBP	down	-1.32	1.7E-16	1.3E-13
	transmembrane signaling receptor activity	GOMF	down	-1.12	2.9E-14	1.1E-11
	transmembrane receptor activity	GOMF	down	-1.11	4.0E-14	1.1E-11
	signaling receptor activity	GOMF	down	-1.06	2.6E-13	5.9E-11

	G-protein coupled receptor signaling pathway	GOBP	down	-1.10	2.4E-13	1.6E-10
	receptor activity	GOMF	down	-0.95	1.1E-11	1.8E-09
	molecular transducer activity	GOMF	down	-0.95	1.1E-11	1.8E-09
	neurological system process	GOBP	down	-1.01	9.2E-12	5.7E-09
	signal transducer activity	GOMF	down	-0.91	4.9E-11	6.9E-09
	odorant binding	GOMF	down	-1.44	3.2E-10	4.0E-08
	intracellular	GOCC	up	0.73	3.4E-10	2.6E-07
	binding	GOMF	up	0.74	3.1E-09	3.5E-07
	intracellular part	GOCC	up	0.63	2.6E-08	1.0E-05
	system process	GOBP	down	-0.72	7.2E-08	4.0E-05
	organelle	GOCC	up	0.57	1.8E-07	4.1E-05
	membrane-bounded organelle	GOCC	up	0.55	2.2E-07	4.1E-05
	intracellular organelle	GOCC	up	0.51	9.8E-07	1.2E-04
	intracellular membrane-bounded organelle	GOCC	up	0.51	8.9E-07	1.2E-04
	cellular metabolic process	GOBP	up	0.55	9.8E-07	5.0E-04
	cytoplasm	GOCC	up	0.46	7.3E-06	8.0E-04
	cellular macromolecule metabolic process	GOBP	up	0.54	1.9E-06	8.7E-04
	palmitoyl-CoA hydrolase activity	GOMF	down	-1.52	2.3E-05	2.3E-03
	integral component of membrane	GOCC	down	-0.43	3.8E-05	3.7E-03
	protein binding	GOMF	up	0.45	5.0E-05	4.7E-03
	macromolecule metabolic process	GOBP	up	0.49	1.1E-05	4.8E-03
	clathrin-coated endocytic vesicle membrane	GOCC	up	1.48	5.9E-05	5.0E-03
	intrinsic component of membrane	GOCC	down	-0.41	8.1E-05	6.2E-03
	organic substance metabolic process	GOBP	up	0.47	1.5E-05	6.3E-03
	LRR domain binding	GOMF	down	-1.38	8.9E-05	7.8E-03
	neurotransmitter receptor activity	GOMF	up	1.91	9.7E-05	7.8E-03
	acyl-CoA hydrolase activity	GOMF	down	-1.37	1.0E-04	7.8E-03
	metabolic process	GOBP	up	0.46	2.3E-05	9.0E-03
	primary metabolic process	GOBP	up	0.46	2.5E-05	9.1E-03
	CoA hydrolase activity	GOMF	down	-1.34	1.3E-04	9.2E-03
	cellular response to ATP	GOBP	up	2.24	2.8E-05	9.6E-03
	clathrin-coated endocytic vesicle	GOCC	up	1.37	1.4E-04	1.0E-02
	organic cyclic compound binding	GOMF	up	0.46	1.6E-04	1.1E-02
	mitochondrial respiratory chain complex IV assembly	GOBP	down	-1.15	4.0E-05	1.2E-02
	mitochondrial respiratory chain complex IV biogenesis	GOBP	down	-1.15	4.0E-05	1.2E-02
	heterocyclic compound binding	GOMF	up	0.45	2.0E-04	1.2E-02
	clathrin-coated vesicle membrane	GOCC	up	1.30	2.1E-04	1.3E-02
	respiratory chain complex IV assembly	GOBP	down	-1.06	6.0E-05	1.7E-02
	nitrogen compound metabolic process	GOBP	up	0.46	6.4E-05	1.8E-02
	membrane part	GOCC	down	-0.36	3.4E-04	2.0E-02
	interferon-gamma-mediated signaling pathway	GOBP	up	1.63	7.5E-05	2.0E-02

	nucleus	GOCC	up	0.37	4.2E-04	2.3E-02
	cytochrome complex assembly	GOBP	down	-0.98	9.7E-05	2.5E-02
	organelle part	GOCC	up	0.34	5.5E-04	2.8E-02
	G-protein coupled nucleotide receptor activity	GOMF	up	2.59	6.7E-04	3.3E-02
	purinergic nucleotide receptor activity	GOMF	up	2.39	5.9E-04	3.3E-02
	G-protein coupled serotonin receptor activity	GOMF	up	2.25	6.2E-04	3.3E-02
	nucleotide receptor activity	GOMF	up	2.39	5.9E-04	3.3E-02
	G-protein coupled purinergic nucleotide receptor activity	GOMF	up	2.59	6.7E-04	3.3E-02
	serotonin receptor activity	GOMF	up	2.22	7.1E-04	3.4E-02
	intracellular organelle part	GOCC	up	0.34	7.6E-04	3.6E-02
	nucleic acid metabolic process	GOBP	up	0.47	1.7E-04	4.1E-02
	establishment of localization	GOBP	up	0.48	1.9E-04	4.4E-02
	enzyme binding	GOMF	up	0.63	1.1E-03	5.0E-02
Patella	detection of chemical stimulus involved in sensory perception of bitter taste	GOBP	down	-1.95	9.7E-08	3.0E-04
	sensory perception of bitter taste	GOBP	down	-1.91	9.8E-08	3.0E-04
	detection of chemical stimulus involved in sensory perception of taste	GOBP	down	-1.87	1.9E-07	3.9E-04
	bitter taste receptor activity	GOMF	down	-2.02	3.8E-07	4.3E-04
	taste receptor activity	GOMF	down	-1.90	9.9E-07	5.6E-04
	sensory perception of taste	GOBP	down	-1.65	1.5E-06	2.3E-03
	olfactory receptor activity	GOMF	up	0.98	9.2E-06	3.5E-03
	detection of chemical stimulus involved in sensory perception of smell	GOBP	up	0.99	7.6E-06	9.3E-03
	endonuclease activity, active with either ribo- or deoxyribonucleic acids and producing 3'-phosphomonoesters	GOMF	down	-1.76	3.5E-05	1.0E-02
	Olfactory transduction	KEGG	up	0.87	7.7E-05	1.6E-02
	dipeptidase activity	GOMF	up	1.67	8.2E-05	1.9E-02
	endodeoxyribonuclease activity, producing 3'-phosphomonoesters	GOMF	down	-1.84	1.3E-04	2.3E-02
	IgG binding	GOMF	up	1.75	1.4E-04	2.3E-02
	sensory perception of smell	GOBP	up	0.92	3.7E-05	3.8E-02
	Taste transduction	KEGG	down	-1.61	4.1E-04	4.4E-02

Note: T1, first trimester; T2, second trimester; T3, third trimester; UCB, umbilical cord blood; GOBP, Gene Ontology Biological Process; GOMF, Gene Ontology Molecular Function; GOCC, Gene Ontology Cellular Component; KEGG, Kyoto Encyclopedia of Genes and Genomes; Coeff, coefficient

Figure 2.3A: Venn diagram of functional annotations from LRPath analysis (q-value<0.05) associated with maternal Pb exposure comparing results from tibia and patella bone.



Chapter 3 Prenatal Lead (Pb) Exposure and Peripheral Blood DNA Methylation (5mC) and Hydroxymethylation (5hmC) in Mexican Adolescents from the ELEMENT Birth Cohort

Abstract

Gestational lead (Pb) exposure can adversely impact offspring health through multiple mechanisms, including epigenomic alterations via DNA methylation (5mC) and hydroxymethylation (5hmC), an intermediate in oxidative demethylation. Most current methods do not distinguish between 5mC and 5hmC limiting insights into their individual roles. The goal of this study is to identify the association of trimester-specific (T1, T2, T3) prenatal Pb exposures with 5mC and 5hmC levels at multiple cytosine-phosphate-guanine (CpG) sites within gene regions previously associated with prenatal Pb exposure (*HCN2*, *NINJ2, RAB5A, TPPP)* in whole blood leukocytes of children ages 11-18 years. Participants from the Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) birth cohorts were selected (n=144) for pyrosequencing analysis following oxidative or standard sodium bisulfite treatment. This workflow directly quantifies total methylation (5mC+5hmC) and 5mC only; 5hmC is estimated by subtraction. Participants were 51% male and mean maternal blood lead levels (BLL) were 6.43±5.16 µg/dL in T1, 5.66±5.21 µg/dL in T2, and 5.86±4.34 µg/dL in T3. 5hmC levels were calculated for *HCN2* (mean±SD, 2.04±4.30%), *NINJ2* (2.66±5.11%)*, RAB5A* (0.64±0.80%), and *TPPP* (0.45±8.01%). 5mC levels were measured in *HCN2* (81.3±9.63%), *NINJ2* (G/C:38.6±7.39%; G/G: 67.3±9.83%)*, RAB5A* (1.40±1.20%), and

TPPP (92.5±8.03%). Several significant associations between BLLs and 5hmC/5mC were reported: T1 maternal BLLs with 5mC in *HCN2* (β=-0.43, p=0.02) and 5hmC in *NINJ2* (β=0.49, p=0.003); T2 BLL with 5mC in *HCN2* (β=0.37, p=0.03) and 5hmC in *NINJ2* (β=0.27, p=0.008); and T3 BLL with 5mC in *HCN2* (β=0.50, p=0.01) and *NINJ2* (β=-0.35, p=0.004) and 5hmC in *NINJ2* (β=0.45, p<0.001). 5mC was negatively correlated with gene expression (Pearson r=-0.5, p-value=0.005) within *NINJ2*, whereas 5hmC was positively correlated (r=0.4, p-value=0.04). These findings suggest there is variable 5hmC in human whole blood and that prenatal Pb exposure is associated with gene-specific 5mC and 5hmC levels at adolescence, providing evidence to consider 5hmC as a regulatory response mechanism to environmental exposures.

Introduction

The Developmental Origins of Heath and Disease (DoHAD) hypothesis [1] postulates that *in utero* exposures, including environmental contaminant exposures such as lead (Pb), can permanently modify an organism's molecular biology, physiology, and metabolism, potentially leading to myriad effects on cognition, growth, maturation, and metabolic risk [2]. Pb is a widely abundant environmental pollutant known to be a potent neurotoxicant, even at low levels. Pregnancy is a vulnerable time period for Pb exposure as both current and past maternal Pb exposure may impact the developing fetus. Animal and human studies have provided evidence for the impact of early-life Pb exposure on the epigenome [3-8].

Epigenetic modifications are mitotically heritable molecular changes that regulate gene expression without altering the underlying DNA sequence. DNA 5-methylcytosine (5mC) is the addition of a methyl group covalently bound to the 5'-carbon of Cytosine; in

mammals this typically occurs on a Cytosine adjacent to a Guanine, referred to as a CpG site [9, 10]. During demethylation, 5mC is oxidized into 5-hydroxymethylcytosine (5hmC), an intermediate of oxidative demethylation that can remain as a stable modification [11, 12]. 5hmC perturbations in the brain have been reported in several early-life neurologic disorders and later-life neurodegenerative disorders in both human and animal studies. [13-16]. Levels of 5hmC vary with tissue, where brain contains the highest levels; blood contains the lowest levels but 5hmC is still detectable at modest levels [17-21]. Recent studies suggest that the functional roles of 5hmC are distinct from 5mC [22]. 5mC and 5hmC undergo dynamic changes during early gestation that disseminate through mitosis to new cells and developing organs, potentially persisting throughout the life span. These modifications are important for gene regulation, including in the nervous system, and have implications for learning and memory from early development, adolescence, and into adulthood [13, 23-25]. Most current methods, including the gold standard sodium bisulfite sequencing, collectively measure 5mC and 5hmC without distinguishing between the two. This has limited our ability to identify whether environmental exposures alter 5mC, 5hmC, or both and what the implications are for early-life neurodevelopment and long-term health.

Pregnancy is a key starting point to investigate the association between environmental contaminants and epigenetic modifications leading to health outcomes in offspring. An important mechanism by which developmental exposures can affect long-term disease risk is through the disruption of normal epigenetic processes, thereby impacting gene regulation and subsequent chronic outcomes [26]. A study prenatally exposing dams, which are genetically invariant mice 93% identical to C57BL/6J strain,

to physiologically relevant doses of Pb (2.1 ppm, 16 ppm, and 32 ppm in water) two weeks prior to mating through lactation until weaning at postnatal day 21 assessed DNA total methylation via pyrosequencing at four Intracisternal A particle (IAP) elements in the brain [27]. IAPs are a class of murine retrotransposons that are environmentally responsive [28]. Prenatal Pb exposure reduced DNA methylation at three of the IAPs in the brain with dose-dependent and sex-specific effects compared to control mice. A study exposing rodents to 0.2% Pb acetate in water postnatally reported hypomethylation and initial transient 8-hydroxy-2'-deoxyguanosine (oxo^8dG) accumulation in the cerebral cortex, a neurodegeneration biomarker [29]. A U.S. prospective human pregnancy cohort, Project Viva, with low Pb exposure (averaging 1.22 ± 0.63 μg/dL in erythrocytes) conducted an epigenome-wide analysis on 268 umbilical cord blood samples to evaluate the association between maternal Pb exposure and DNA methylation, and identified sex-specific differentially methylated CpG sites [7]. Using a similar epigenome-wide approach but with a moderately to highly exposed population, we identified differentially methylated genes in 89 umbilical cord blood leukocyte DNA samples from the Early Life in Mexico to Environmental Toxicants (ELEMENT) cohort that varied by trimester of exposure [6]. Although these and several other studies provide evidence for associations between Pb exposure and DNA methylation profiles in humans and animals, only total methylation was assessed.

Only one study has considered intermediates (5hmC) in these adverse environmental associations with prenatal Pb, which may have implications for understanding the association between prenatal exposures and adverse health outcomes. That study provided some evidence for associations between prenatal Pb

and 5hmC in umbilical cord blood at the regional level through a modified epigenome-wide method called hMeDIP-450K chip [30]. They identified both sex-independent and sex-specific differentially methylated and hydroxymethylated regions, where sex-dependent associations were more common in 5mC compared to 5hmC. Mechanistically, Pb-induced oxidative stress results in the accumulation of α-ketoglutarate (α-KG) [31], a co-factor for ten-eleven translocation (TET) enzymes which are involved in the oxidation of 5mC to 5hmC [32, 33]. Thus, Pb may increase activity of TET enzymes and increase 5hmC across the genome [32].

We hypothesized that prenatal Pb exposure would alter epigenetic programming of 5mC and 5hmC within genes involved in neurological function, and that this can be detected in adolescent-aged samples. We leveraged the ELEMENT longitudinal birth cohort with rich data on prenatal Pb exposure and offspring follow-up through adolescence, including whole blood leukocyte DNA and prenatal Pb exposure biomarkers, to investigate whether Pb is associated with programming of neurocognitive-related genes between the ages of 11-18 years in offspring whole blood leukocytes. We utilized oxidative-bisulfite (oxBS) pyrosequencing to profile both 5mC and 5hmC in adolescent blood leukocyte DNA at four neurocognitive-related genes *HCN2*, *NINJ2, RAB5A,* and *TPPP* that were associated with prenatal Pb exposure in ELEMENT in our previous epigenome-wide study of total methylation in cord blood samples [6].

Methods

Study population

Participants are from the second and third cohorts of the ELEMENT project, a series of longitudinal birth cohorts originally designed to investigate the influence of Pb exposure – *in utero* and in childhood – on child growth and neurodevelopment. Mothers were recruited between 1997-2000 (second cohort) and 2001-2005 (third cohort) from the Mexican Social Security Institute hospital in Mexico City. Eligibility and exclusion criteria are as previously described [34, 35]. Data collected include sex, gestational age, household socioeconomic status, anthropometry, and other environmental exposures at multiple follow-up visits from infancy through adolescence. For the current study, 144 of the ELEMENT participants were selected that were followed-up through the 2015 study visit and had an adolescent blood sample for DNA isolation (**Figure 3.1**). Participants with prenatal and adolescent Pb measures and/or previous cord blood DNA methylation analysis were prioritized for the adolescent epigenetic analysis [6].

Prior to participation, all mothers were informed about the study; those who agreed to participate read and signed a letter of informed consent about the original study. Children also provided informed consent prior to participation in the adolescent follow-up visits. The research protocol and all amendments to the study were approved by the Ethics Committees of the National Institutes of Public Health of Mexico, participating hospitals, and the Internal Review Board at participating institutions including the University of Michigan.

Pb exposure assessment and genomic DNA isolation

Cohort 2 maternal venous blood lead levels (BLLs) from each trimester were measured using inductively coupled plasma mass-spectrometry (ICP-MS, Thermo Finnigan, Bremen, Germany) at the University of California, Santa Cruz, as described previously [36]. Cohort 3 trimester-specific maternal BLLs were measured using graphite furnace atomic absorption spectrometry (instrument model 3000; PerkinElmer, Norwalk, CT, USA) at the Trace Metal Laboratory of the American British Cowdry Hospital. Perinatal maternal bone Pb levels were also measured in the left patella (trabecular bone) and mid-shaft of the left tibia (cortical bone) 1-month post-partum as an indicator of cumulative Pb exposure during pregnancy using a spot-source ^{109}Cd K-shell X-ray fluorescence (K-XRF) instrument. The technical specifications and validation of this instrument are described in detail elsewhere [37]. Tibia and patella bone Pb measures were dropped if their associated uncertainty levels were greater than 10 μg/g (n=1) and 15 μg/g (n=0), respectively. Next, any negative tibia (n=27) and patella (n=20) bone Pb measurements were re-coded as positive values with random numbers in a uniform distribution between 0 and the limit of detection. Whole blood samples were collected during the 2015 follow-up visit conducted when the children were between the ages of 11-18 years old, and blood was stored frozen at -80^{o}C. DNA was isolated from blood leukocytes using Qiagen kits and standard protocols for blood DNA isolation. Nucleic acid yield and purity were assessed first using a NanoDrop spectrophotometer (ThermoFisher Scientific), and double-stranded DNA was also quantified via a Qubit fluorometer. All DNA samples were stored at -80^{o}C.

Candidate gene selection

CpG sites that mapped back to four genes relevant to neurological function were selected as candidates from an epigenome-wide study of prenatal Pb exposure conducted with a subset of ELEMENT children in umbilical cord blood [6] and adolescent whole blood DNA (unpublished): *HCN2* (cg06657917)*, NINJ2* (cg19692784, cg14911689, cg05578102)*, RAB5A* (cg17138393)*,* and *TPPP* (cg25353752; probe IDs from the Infinium MethylationEPIC BeadChip)*. RAB5A* DNA methylation in umbilical cord blood [6] and *TPPP* DNA methylation (unpublished) in adolescent whole blood were inversely associated with T1 maternal BLLs and were selected for the current targeted analysis. *HCN2* and *NINJ2* were selected because T1 BLLs were associated with greater than 5% change in methylation (unpublished; hypermethylated in *HCN2* and hypomethylated in *NINJ2*) in both umbilical cord blood and adolescent whole blood leukocytes, and these genes are involved in gestational neurological and/or cognitive development, for which mutations or disruptions in function have been associated with neuronal activity [38], and neurite outgrowth and regeneration [39]. *EXT1* and *LRFN1* were additionally reported as significant in the original umbilical cord blood epigenome-wide study but were not included in this study because 1) we were unable to design primers to bind to the desired region in *EXT1* and 2) *LRFN1* was not associated with first trimester BLLs, which was the original research question during the development of this study.

DNA 5mC and 5hmC quantification

Pyrosequencing primers for *HCN2*, *NINJ2*, *RAB5A*, and *TPPP* were designed using the PyroMark Assay Design Software 2.0 Methylation Analysis (CpG) Assay

(**Appendix Table 3.1A**). Primers were designed to target a specific CpG site within a region reported to be differentially methylated with first trimester Pb exposure in umbilical cord blood and/or adolescent whole blood (see *Candidate gene selection*). ELEMENT genomic DNA samples were oxidative and/or bisulfite treated according to the NuGen TrueMethyl oxBS Module protocol (Cat. #0414-32) and Zymo EZ DNA Methylation kit (Cat. #D5003). Briefly, 1µg of input genomic DNA was dissolved in nuclease-free water to 50µl, and each genomic DNA sample was divided into two aliquots [40]. Each aliquot underwent independent, parallel treatments and were either oxidative bisulfite converted with the NuGen TrueMethyl oxBS Module or Zymo EZ DNA Methylation kit. The yield and purity of treated samples were quantified using a NanoDrop spectrophotometer.

The target loci within *HCN2*, *NINJ2*, *RAB5A*, and *TPPP* were amplified in both bisulfite and oxidative bisulfite converted samples. PCR products were verified using the QIAxcel automated DNA electrophoresis. DNA methylation levels were quantified using the PyroMark Q96 ID instrument (Qiagen). Targeted pyrosequencing captured six CpG sites within a CpG island of *HCN2*, eight within a DNase hypersensitivity region of *NINJ2*, eleven within a DNase hypersensitivity region in the first exon of *RAB5A*, and four within the third exon of *TPPP*. CpG site #6 with *NINJ2* was dropped since 90% of samples failed at this location, leaving seven CpG sites for *NINJ2* from downstream analysis. Sequencing the bisulfite converted samples quantifies the total level of 5mC + 5hmC, while sequencing the oxidative bisulfite treated samples quantifies total levels of 5mC. Thus, 5hmC levels were quantified by subtracting the results from the oxidative bisulfite converted samples (5mC) from the bisulfite converted sample (5mC + 5hmC). It

should be noted that because 5hmC is based on a calculation, the difference can sometimes be negative at sites with zero or low levels of methylation as a consequence of random noise [41, 42]. It should be noted that less than 1.4% of all 5hmC values were less than -10%. For quality control, plates were run with Qiagen EpiTect bisulfite converted unmethylated (0%) and methylated (100%) human methylation standards (Cat. #59665 & #59655). OxBS triplicates of eight samples were included for quality control. OxBS triplicate standard deviations of *NINJ2* (variably methylated) and *TPPP* (highly methylated) in quantified 5mC averaged 2.11 and 2.28 respectively at each CpG site captured. Measures of 5mC were precise with <10% coefficient of variation, whereas the measures of methylation utilizing the standard bisulfite method was more precise with <5% coefficient of variation. DNA samples were randomized across experimental batches which consisted of four plates for each gene. Paired oxBS and BS conversion samples were always in the same batch. All data that failed internal quality control checks within the PyroMark software were excluded from analysis (*HCN2*: 5mC n=3, 5hmC n=7; *NINJ2*: 5mC n=7, 5hmC n=9; *RAB5A*: 5mC n=1, 5hmC n=1; *TPPP*: 5mC n=2, 5hmC n=3).

Estimates of cell-type composition (CD4+ and CD8+ T lymphocytes, B cells, natural killer [NK] cells, monocytes, granulocytes) for each sample were performed using an established method based on adult cell-type specific differentially methylated regions using data from the Infinium EPIC array for each sample [43].

A methylation quantitative trait locus (MeQTL) was identified within *NINJ2* and was found to be correlated with a SNP (C/G; rs34038797) within the pyrosequenced region [44]. Genotyping was performed on genomic DNA from all 144 samples using the

PyroMark Q96 ID instrument (Qiagen). Pyrosequencing SNP primers for *NINJ2* were designed using the PyroMark Assay Design Software 2.0 Genotyping (SNP) Assay (**Appendix Table 3.1A**). Primers were designed to target the rs34038797 SNP.

Gene expression analysis via RNA sequencing

For a subset of participants (n=70), next generation sequencing of RNA ("RNA-seq") was conducted to obtain relative expression data for all genes and utilized data from the four genes that are the focus of this paper. Following collection of whole blood into EDTA-containing tubes, white blood cells were extracted, preserved in RNALater, and stored frozen (-80^{o}C) until further processing. RNA was isolated via the All-Prep kit (Qiagen). Quality and quantity were assessed via a Bioanalyzer Tapestation (Agilent). Libraries were prepared with Universal Plus mRNA-Seq with Human globin AnyDeplete (NuGEN Technologies, Inc.) which removes globin transcripts that are highly abundant in blood samples. Library preparation and sequencing were performed at the University of Michigan Advanced Genomics Core. Paired-end 50 cycle sequencing on an Illumina HiSeq 4000 was performed. Quality of the raw reads data for each sample was checked using FastQC (version 0.11.3). The Tuxedo Suite software package was used for alignment [45-47]. Briefly, reads were aligned to the reference mRNA transcriptome (hg19) using TopHat (version 2.0.13) and Bowtie2 (version 2.2.1.) followed by a second round of post-alignment quality control in FastQC, which allows at most three mismatches. One sample was dropped due to low alignment rates. All samples used in downstream analysis (n=69) had at least 16.8 million good quality aligned reads with alignment rates averaging 60%. Prior to analysis, read counts were normalized by the trimmed mean of M-values method [48].

Statistical analysis

All statistical analyses were performed in the R Project for Statistical Computing (version 3.6.1). Summary statistics were first calculated. Pb variables analyzed include maternal BLLs at each trimester (T1, T2, T3), bone Pb levels in maternal patella and tibia, and BLL measured at the adolescent follow-up visit, which were all treated as continuous variables. Bivariate analyses between Pb exposure variables and covariates (e.g., cell-type proportions, adolescent sex, maternal age at birth and adolescent age at time of sample collection, pyrosequencing plate (i.e., batch), height-for-age z-score, BMI-for-age z-score, weight, etc.) was performed. Potential confounding variables that were associated with both prenatal Pb and DNA methylation - adolescent age and adolescent BLL - were identified for inclusion in final statistical models of site-specific DNA methylation data. While not associated with Pb, sex and experimental plate were also included in statistical models due to their biological effect on DNA methylation and effect on the technical measurement of DNA methylation, respectively. A random intercept for each participant was included in all models to account for autocorrelation from matched 5mC and 5hmC percentages for each individual. Lastly, in models of *NINJ2* methylation, C/C individuals had 0% regional methylation as expected since the SNP converts the CpG site to a CpC site, resulting in the inability to methylate the locus and ensuing regional methylation changes. Thus, C/C individuals were dropped from analysis and genotype (G/G or G/C) was included as a covariate.

A mixed-effects model was run treating 5mC and 5hmC values as repeated measures of a single outcome variable – DNA methylation, given that 5mC and 5hmC are both measured at each CpG site and the values for these two marks are dependent

upon each other both biologically and statistically [49]. To determine if Pb exposure modifies the balance between the DNA modification categories (5mC and 5hmC), an interaction term between prenatal Pb measures and a dichotomous variable signifying whether the outcome measure is 5hmC or 5mC was included in the statistical model. Since there was evidence for an interaction between Pb and type of DNA methylation, we next used separate mixed-effects regression models treating CpG sites as repeated measures to estimate associations between prenatal Pb and 5mC and associations between prenatal Pb and 5hmC, separately. To assess potential sex-specific effects on the association between prenatal Pb and DNA 5mC and 5hmC, identical models were run as described above but stratified by sex. The *lme4* and *lmerTest* packages within the statistical program R were used for these analyses and a p-value<0.05 was considered significant. Finally, RNAseq normalized read counts of *HCN2, NINJ2, RAB5A,* and *TPPP* were log-transformed and compared to 5mC% and 5hmC% from pyrosequencing. We used Pearson's product-moment correlation to quantify the strength of the relationship between these expression and epigenetics. P-values<0.05 were considered significant. The *ggplot2* R package was used to plot results.

Due to outliers in many of the Pb biomarkers, a sensitivity analysis was performed. In this analysis, we re-ran the models excluding outliers for BLLs or bone Pb measures. Outliers were defined as ±3D from the mean. We excluded 4, 5, 1, 3 and 5 outliers that were all 3 SD greater than the means for T1 BLL, T2 BLL, T3 BLL, tibia bone and patella bone Pb, respectively. We compared results with and without the outliers.

Results

Population parameters and phenotypic data

Among the 144 children in the analytic sample, 73 (51%) were male (Table 1). The mean age of the offspring was 14.0 years, ranging from 11.1 years up to 17.7 years of age. The mean maternal BLLs averaged over all three trimesters was 5.98 (SD=4.34) μg/dL, with a first trimester (T1) mean of 6.43 (SD=5.16) μg/dL, second trimester (T2) mean of 5.66 (SD=5.21) μg/dL, and a third trimester mean of 5.86 (SD=4.32) μg/dL. Maternal BLLs between the trimesters were highly correlated ($r>0.67$, $p<10^{-6}$) according to a Spearman's rank-order correlation test. Bone Pb measures represent long-term Pb accumulation since the half-life of Pb in bone can be decades. Average Pb concentration measured in one-month post-partum maternal patella was 11.4 (SD=9.45) μg/g and tibia was 9.19 (SD=6.88) μg/g. Current adolescent BLL was 3.29 (SD=4.44) μg/dL, which was moderately correlated ($r>0.34$, $p<0.0001$) with maternal trimester-specific Pb exposures. Genotyping of study participants at rs34038797 SNP revealed 27 individuals with G/G genotype, 83 G/C, and 34 C/C. Participants included in this analysis were compared with all ELEMENT mother-infant pairs from the same cohorts (2 and 3). Characteristics of offspring and Pb biomarker concentrations were not statistically different between the subset included in this analysis and the entire sample (**Table 3.1**) with the exception of adolescent age at follow-up which was slightly lower in the subset.

5mC and 5hmC within candidate regions

To measure the levels of DNA 5mC and 5hmC at *HCN2, NINJ2, RAB5A,* and *TPPP* loci, we performed parallel BS and oxBS conversion of DNA samples from the

adolescent ELEMENT samples. Both 5mC and 5hmC were measured at each gene loci in all adolescent whole blood DNA samples. The mean percentages of 5hmC across genetic regions were measured at 2.04% (SD=4.31) in *HCN2,* 3.00% (SD=6.37) for G/C samples in *NINJ2* and 1.47% (SD=5.86) in G/G *NINJ2,* 0.64% (SD=0.80) in *RAB5A*, and 0.45% (SD=8.01) in *TPPP* (**Figure 3.2**, **Appendix Table 3.2A**). Percent measurements of 5hmC in blood at each locus were detectable, but much lower than for 5mC, with 13 participants displaying average 5hmC measures at or above 10% in at least one gene. 5mC was measured at 81.3% (SD=9.63) in *HCN2,* 38.6% (SD=7.39) for G/C samples *NINJ2* and 67.3% (SD=9.83) in G/G *NINJ2,* 1.41% (SD=1.21) in *RAB5A* and 92.5% (SD=8.03) in *TPPP*.

Association of prenatal Pb with 5mC and 5hmC

Next, an interaction term was used to determine if prenatal Pb exposure is associated with 5mC and 5hmC in the same or a different manner. In a repeat measures model with the paired 5mC and 5hmC measures as outcomes, several interaction terms were statistically significant. In one example, as prenatal Pb exposure increases, there was a decrease in the slope between 5mC and 5hmC in whole blood, signifying a shift towards a greater proportion of 5hmC compared to 5mC in *NINJ2.* This shift towards 5hmC was reflected in the significant negative interaction terms between DNA modification category and Pb exposure ($p<0.001$ for all Pb exposure measures) (**Appendix Table 3.3A**). Similarly, the 5mC and 5hmC proportions in *HCN2* shift towards increased 5mC at higher Pb exposure measures when analyzing T1 BLLs, T2 BLLs, T3 BLLs, and patella bone Pb (not tibia bone Pb), which was reflected in the significant positive interaction terms ($p\leq0.001$). Interestingly, *TPPP* 5mC and 5hmC

proportions shifted towards 5mC at higher patella bone Pb levels ($p<0.03$) but shifted towards 5hmC at higher tibia bone Pb measures ($p<0.03$). There was no significant interaction between Pb and type of DNA methylation and also no significant main effect of Pb in the model of *RAB5A.*

Since there was evidence for differences in the association between Pb and DNA methylation by type of methylation at several genes, the association between each Pb exposure biomarker and 5mC or 5hmC at each gene was tested separately. For each unit increase (1 μg/dL) in maternal BLL during T1, there was a 0.43 (95% CI: 0.07, 0.78) increase in % 5mC in *HCN2* ($p=0.02$) and a 0.49 (95% CI: 0.17, 0.80) increase in % 5hmC within the *NINJ2* locus ($p=0.003$) (**Figure 3.3**, **Appendix Table 3.3A**). DNA 5mC levels within *HCN2* demonstrated a positive association with T2 BLLs ($\beta=0.37$ (95% CI: 0.04, 0.69), $p=0.03$), but within *NINJ2* it was 5hmC levels that were associated with T2 BLLs ($\beta=0.27$ (95% CI: 0.07, 0.47), $p=0.008$) (**Appendix Table 3.3A**). Similarly, T3 maternal BLLs were associated with an increase in *HCN2* 5mC ($\beta=0.50$ (95% CI: 0.10, 0.90), $p=0.01$) but a decrease in *NINJ2* ($\beta=-0.35$ (95% CI: -0.60, -0.11), $p=0.004$). T3 BLL were also associated with an increase in *NINJ2* 5hmC levels ($\beta=0.45$ (95% CI: 0.21, 0.68), $p<0.001$) (**Figure 3.3, Appendix Table 3.3A**). There were no observed significant associations between trimester-specific Pb measures and 5mC or 5hmC of *RAB5A* or *TPPP.* We next investigated whether patella or tibia bone Pb measures of cumulative gestational exposure were associated with either 5mC or 5hmC at each locus. A 1 μg/g increase in patella bone Pb was associated with a significant increase in 5hmC ($\beta=0.17$ (95% CI: 0.07, 0.27), $p<0.001$) and a non-significant decrease in 5mC ($\beta=-0.27$ (95% CI: -0.56, 0.03), $p=0.08$) in *NINJ2* (**Appendix Table 3.3A**). However,

patella Pb and tibia Pb were not significantly associated with 5mC or 5hmC in *HCN2*, *RAB5A*, or *TPPP.*

The results of the sensitivity analysis excluding outliers for trimester-specific BLLs and bone Pb measures demonstrated associations with DNA 5mC and 5hmC in the same direction for each exposure-outcome relationship that were statistically significant in models including all subjects (**Appendix Table 3.3A**). After outliers were removed, associations remained significant for T1 BLLs and 5mC in *HCN2*, and for T1, T2, and T3 BLLs and patella bone Pb and 5hmC in *NINJ2*, while associations of T2 and T3 BLLs with 5mC in *HCN2* remained positive but were no longer statistically significant. However, the estimate for T3 BLLs and 5mC in *NINJ2* changed from a significant inverse association to a null association (β=–0.01; 95% CI: –0.79, 0.77) after one outlier for this exposure was excluded from the analysis.

Sex-specific estimates

To investigate whether trimester-specific or cumulative (bone) Pb exposure biomarkers were associated with male or female-specific alterations in 5mC and/or 5hmC at *HCN2, NINJ2, RAB5A,* and *TPPP* loci (**Table 3.2**), we performed sex-stratified models. For *HCN2*, associations between 1 μg/dL increases in T2 and T3 BLLs and 5mC were positive for females and null for males (e.g., T2: β=0.82 (95% CI: 0.25, 1.38) vs. β=0.02 (95% CI: –0.41, 0.45), respectively) while associations with 5hmC were negative for females and positive for males (e.g., T3 BLLs, β=-0.30 (95% CI: -0.62, 0.03) vs. β=0.12 (95% CI: -0.11, 0.36), respectively). Associations with 5mC and 5hmC in *HCN2* were not notably different by sex. For *NINJ2*, T2 BLLs were positively associated with 5hmC in females, while the association was close to the null for males

(β=0.48 (95% CI: 0.10, 0.86) vs. β=0.07 (95% CI: –0.20, 0.34), respectively). Associations of 5mC in *NINJ2* with T1 BLLs and patella Pb were negative for females and close to the null for males, but confidence intervals overlapped; otherwise, effect estimates were similar by sex. Consistent with the results for *RAB5A* in males and females combined, associations with 5mC and 5hmC were consistently null for both groups. For *TPPP*, patella Pb was positively associated with 5mC and negatively associated with 5hmC in males (β=0.16 (95% CI: 0.05, 0.27) and β=-0.14 (95% CI: -0.29, 0.02), respectively) while corresponding estimates were close to the null in females (β=-0.04 (95% CI: -0.16, 0.08) and β=0.04 (95% CI: -0.08, 0.15), respectively). The sex-stratified sensitivity analysis results excluding outliers for trimester-specific BLLs and bone Pb measures demonstrated effect estimate changes (**Appendix Table 3.4A**). In *HCN2*, the magnitude of the association between 1 μg/dL increases in T1 BLLs and 5mC became more positive in females by 0.42% (β =0.83 (95% CI: 0.08, 1.59), whereas males remained unchanged. All other associations with 5mC and 5hmC in *HCN2* were not notably different in the sensitivity analysis. In *NINJ2,* T1 BLLs were more negatively associated with 5mC by 0.64%, whereas the association among females was diminished (β=-0.66 (95% CI: -2.01, 0.68) and β=0.04 (95% CI: (-1.37, 1.43), respectively). T2 BLLs changed directions to a positive association with 5mC in females (β=0.30 (95% CI: -0.88, 1.48)), whereas the male magnitude with 5hmC increased by 0.31% (β=0.38 (95% CI: -0.22, 0.99)). Patella Pb gained a negative association with 5mC and positive with 5hmC (β=-0.52 (95% CI: -1.03, -0.02), β=0.26 (95% CI: 0.08, 0.44), respectively); otherwise, effect estimates were similar when outliers were included. Consistent with the results with *RAB5A* all other models,

associations with 5mC and 5hmC were consistently null. Similarly, *TPPP* sensitivity analysis by sex did not differ in magnitude from original sex-stratified models.

5mC and 5hmC correlations with gene expression

To compare quantified methylation levels with gene expression (normalized read counts), we compared DNA 5mC and 5hmC measures from the pyrosequencing method with a subset of individuals who had available RNA-seq data (used as normalized read counts; **Figure 3.4** and **Appendix Figure 3.1A**). DNA 5mC was negatively correlated with *NINJ2* expression (R=-0.5, p-value=0.005), whereas DNA 5hmC was positively correlated with *NINJ2* expression (R=0.4, p-value=0.04) (**Figure 3.4**). *RAB5A* had similar directionality but was not statistically significant; 5mC was negatively correlated with expression (r=-0.1, p-value=0.4) and 5hmC was positively correlated (r=0.02, p=0.9). *HCN2* (5mC: r=0.1, p-value=0.5; 5hmC: r=0.05, p-value=0.7) and *TPPP* (5mC: r=0.1, p-value=0.5; 5hmC: r=0.08, p-value=0.6) did not have statistically significant correlations.

Conclusion

Prenatal and early-life Pb exposures have been associated with altered DNA methylation patterns, which serve as potential mechanistic links among Pb-induced health effects [3, 5, 7, 8, 27, 50-52]. The majority of studies, however, quantify total DNA methylation together, and fail to distinguish between 5mC and 5hmC. These include those based on sodium bisulfite conversion of DNA, which is unable to discriminate between 5mC and 5hmC with important implications for the interpretation of published epigenetic studies focused on Pb exposure to date. To our knowledge, this is the first epigenetic study to investigate the independent associations of DNA 5mC and 5hmC in

human blood samples with prenatal Pb exposure. Our study was strengthened by the inclusion of multiple biomarkers of early-life Pb exposure.

First, by utilizing oxBS-pyrosequencing, we provide evidence for the presence of variable 5hmC in human whole blood. Second, we estimated the effect of prenatal Pb exposure on both 5mC and 5hmC of four genes with neurological functions selected from an epigenome-wide study of prenatal Pb exposure (*HCN2, NINJ2, RAB5A,* and *TPPP)*. We observed associations between 5mC with T1, T2, and T3 BLLs in *HCN2,* in addition to T3 BLL and patella bone Pb in *NINJ2*, as well as show that T1, T2, T3 BLLs and cumulative gestational Pb exposure measured in patella bone are associated with 5hmC levels in *NINJ2*. This supports the idea that prenatal Pb exposure stably alters gene-specific 5mC and 5hmC levels that can be detected into adolescence. Further, male- and female-specific associations of prenatal Pb exposure with both 5mC and 5hmC were not notably different by sex. Lastly, we identified correlations between 5mC and 5hmC with gene expression in one of the genes, *NINJ2*.

Our study provided estimates of 5mC and 5hmC in whole blood at four genes with varying methylation profiles. Levels of 5hmC in blood were relatively low with wide variability between individuals, consistent with prior research which estimates the highest levels of 5hmC are in brain tissue with lower levels estimated in tissues such as blood [19-21]. A cross-sectional study of female neonates (n=48) and French-Canadian women aged 25–30y, 70–75y, and ≥90y (n=50 in each age group) reported that mean 5hmC levels were highest in cord blood DNA and lowest in peripheral blood DNA from women in the two oldest groups, suggesting declining levels with age [53]. Of note, we quantified average 5hmC levels as high as 10% within *HCN2, NINJ2,* and *TPPP* genes

for 13 individuals. Although, we also detected negative 5hmC values, which is an inherent limitation of the oxBS subtraction method when assessing gene-specific 5hmC via pyrosequencing. While brain is the tissue of interest in terms of Pb toxicity, surrogate epigenetic measures such as in blood are typically necessary in longitudinal epidemiological studies. Research has shown that there are widespread changes in 5hmC occurring during human brain development, with sex-differences in 5hmC levels in fetal brain [24]. Whether 5hmC levels in blood correlate with crucial 5hmC patterning in brain and if so, at which genes, is currently unknown but needs to be characterize to understand the implications of exposure-5hmC associations in surrogate tissues. [54]. The Toxicant Exposures and Responses by Genomic and Epigenomic Regulators of Transcription (TaRGET II) consortium is one research program that is conducting analyses of 5mC, 5hmC, and more in target and surrogate tissues (including brain and blood, respectively) from animals prenatally exposed to common environmental toxicants in order to understand the role that environment plays in disease susceptibility as a function of tissue-specific epigenomic perturbations [55].

By using adolescent-age DNA methylation profiles, we observed evidence that Pb exposure may modify the balance between 5mC and 5hmC within total methylation. We detected a positive association between trimester-specific BLLs, but not bone Pb, with 5mC in *HCN2*. Further, increasing prenatal Pb exposure was associated with increases in 5hmC but decreases in 5mC in whole blood in *NINJ2*. For example, a 1μg/dL increase in T3 BLLs was associated with lower 5mC but higher 5hmC in *NINJ2.* Although, the association between T3 BLLs and 5mC was null after an outlier was excluded. Metal exposures, such as Pb, cause increased oxidative DNA damage which

inhibits the ability of methyltransferases to interact with DNA, and these can result in hypomethylation at some loci [56-59]. TET proteins are involved in the oxidation of 5mC to 5hmC, and free radicals are known to interact with these proteins [32, 33]. Pb-induced oxidative stress inhibits α-ketoglutarate dehydrogenase in the mitochondria resulting in the accumulation of α-ketoglutarate (α-KG), a co-factor for TET enzymes [31]. Thus, we hypothesize that Pb exposure would increase activity of TET enzymes, resulting in genome-wide increases in 5hmC levels. The findings with *NINJ2* reflect the hypothesis where increased 5hmC was associated with all Pb exposure measures, with or without Pb biomarker outliers included. In contrast, we identified a shift towards higher 5mC as Pb exposure increased for *HCN2*. This may be explained by interacting proteins that are required for HCN channel gating and kinetics. More studies are needed in understanding the interactions between DNA methylation and DNA oxidation.

While both males and females are vulnerable to the adverse effects of Pb exposure, there is significant evidence that sex can influence the severity of Pb neurotoxicity [50, 60, 61]. We observed some evidence for sex-specific associations, though based on magnitude, direction and CI overlap between male and female-specific effect estimates, it is not entirely conclusive. Our study would benefit from a larger sample size in order to draw such conclusions around sex-effects. These data are inconsistent with previous studies in humans and mice in which more Pb-associated methylated CpG sites were identified in females compared to males [50, 62]. It is known that global DNA methylation profiles are dimorphic by sex when assessed in fetal, child, and adult human brain tissue [63] and influenced by hormones when assessed in brain tissue in rats

[64]. Thus, these interactions may influence an individual's susceptibility to the effects of Pb.

Within the a different cohort of the broader ELEMENT study, Sen et al., 2015a reported associations between prenatal Pb exposure on 5hmC in umbilical cord blood [30]. Umbilical cord blood from 24 male and 24 female newborns were randomly selected from the 1st (<1.74 µg/dL) and 4th (>3.77 µg/dL) quartiles of Pb exposure, where 25% of their samples (5 males and 7 females) had BLLs in umbilical cord blood at or above 5 µg/dL. Even though their sample size was relatively low, they observed significant associations between Pb and 5hmC as well as 5mC profiles measured with a modified epigenome-wide method, called hMeDIP-450K chip. Further, they categorized the differentially hydroxymethylated (DhMR) and differently methylated (DMR) regions into sex-independent and sex-specific DMRs. However, sex-dependent associations were more common in 5mC compared to 5hmC profiles. They concluded that differential 5mC was a better predictor for sex-specific effects of Pb exposure, whereas our stratified analysis identified 5hmC as the better predictor. None of their statistically significant DMRs or DhMRs overlapped with our candidate genes. These discrepancies could be due to a variety of reasons. 1) Our study benefitted from a larger samples size with greater variability of prenatal Pb exposure levels. 2) We assessed prenatal Pb on a continuous scale rather than low versus high Pb exposure. 3) Our Pb estimates were taken from prenatal exposures measured in the mothers during pregnancy versus an at birth measure in umbilical cord blood. Nonetheless, their effect sizes and directions were relatively similar for both 5hmC and 5mC associations with Pb, identifying both hyper- and hypo- changes in both DNA modifications. We both conclude that the

number of human studies assessing 5hmC is limited and that our studies provide promise for an increased understanding of Pb-induced epigenetic regulation in humans.

Though we were limited to analyzing whole blood samples, results from this research provide preliminary evidence that Pb exposure may impact regulation of both 5mC and 5hmC. Challenges in current developmental toxicology studies include determining whether persistent epigenetic alterations are due to global changes across all cells, altered cell-type proportions, or changes in specific cell types [65, 66]. At this point in time, the stability of 5hmC within different blood cell-types is unknown [43]. Therefore, it is possible that associations between prenatal Pb and gene-specific 5hmC alterations documented here are simply reflecting blood cell-type proportion shifts. The associations reported here between Pb and blood DNA methylation could be under or overrepresenting potential effects on the target tissue of interest for Pb toxicity – brain. However, additional studies have shown DNA methylation conservation among different tissue and cell-types [67], suggesting that differential methylation from whole blood can be a valid biomarker for tissues of interest at many genes. Future studies should consider cell and tissue type-specific impacts of perinatal Pb exposure to explain mechanisms and reveal biological pathways underlying the adverse outcomes of Pb exposure. Further, larger studies should incorporate oxBS based methodologies to study the sex-specific impact of Pb and other environmental exposures on 5hmC and 5mC or employ methods that directly measure 5hmC (i.e. HMeDip-seq [68]) to avoid negative measurements. Our study had a relatively wide range of Pb exposures, giving us the opportunity to investigate associations relevant to populations with a range of exposures. Although this project measures 5mC and 5hmC levels in only a few selected

loci, it demonstrates the utility of oxBS pyrosequencing as a quantitative and high-throughput method to estimate 5hmC levels at specific regions that does not require antibodies.

In terms of gene regulation, we were able to detect statistically significant correlations within *NINJ2*, where increases in gene expression were associated with hypomethylation and hyper-hydroxymethylation. This follows previous research that has shown that increased levels of 5mC are associated with decreased transcription factor binding at promoter/enhancer sites and suppression of transcription [69]; whereas, 5hmC is associated with increases in gene expression [70, 71]. We were unable to detect these same correlations within *HCN2, RAB5A,* and *TPPP.* Despite nearly half the samples having RNA available for RNA-seq, the use of these samples allowed for direct measurement of expression of each gene analyzed within this study. To further explore the idea that altered 5hmC in human blood impacts gene regulation, future studies should include a larger sample size, as well as investigate expression in individual cell-types utilizing technologies such as single-cell RNA-seq.

In conclusion, these data suggest that 1) prenatal Pb exposure differentially influences 5mC and 5hmC and does not seem to vary by sex, and 2) 5hmC is present and detectable in whole blood samples at variable levels. These modification-specific associations would not have been detected using methods based on standard sodium bisulfite treatment, demonstrating the utility of incorporating oxBS treatment in cohort studies. Prenatal Pb exposure results in a shift from 5mC to 5hmC in *NINJ2,* with the opposite shift seen in *HCN2* (5hmC to 5mC), and these relationships were detected using all blood Pb biomarker measures. Prenatal Pb exposure has been previously

associated with a decrease in IQ and adverse effects on neurobehavioral outcomes, but whether epigenetic mechanisms contribute to these long-term effects is not well characterized. Given the potential importance of Pb-induced DNA methylation perturbations on processes related to early neurodevelopment and potential late-onset delays (i.e., adulthood) in cognition [52], careful examination of Pb-induced 5mC and 5hmC alterations may eventually improve our knowledge of the epigenetic pathways involved in neurodevelopment. Our results suggest the prenatal Pb exposure may modify both 5mC and 5hmC, and the relationship between these changes and neurodevelopment outcomes merits further study. This study and others are providing additional evidence around DNA 5hmC as being an environmentally responsive modification [17, 30]. Altogether, it is vital for environmental studies to consider 5hmC as a regulatory response mechanism to environmental exposures.

Acknowledgements

I would like to thank my coauthors for their valuable input and feedback on this published manuscript: Jaclyn M Goodrich, Maritsa Solano-González, Adriana Mercado-García, Howard Hu, Martha M Tellez-Rojo, Karen E Peterson, and Dana C Dolinoy. The authors acknowledge the research staff at the American British Cowdray Hospital in Mexico City for providing research facilities. We thank the mothers and children for participating in the study. This study was made possible by U.S. Environmental Protection Agency (US EPA) grants RD834800 and RD83543601 and National Institute for Environmental Health Sciences (NIEHS) grants P20 ES018171, P01 ES02284401, R01 ES007821, R01 ES014930, R01 ES013744, R01ES021446, R24-ES-028502, 1U2C ES026553, and P30 ES017885. This work was also supported by University of Michigan (UM) Genome Science Training Grant T32 HG000040 (CR). This study was also supported and partially funded by the National Institute of Public Health/Ministry of Health of Mexico.

Manuscript in Review

This chapter is a slightly modified version of a manuscript in review.

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Tables and Figures

Table 3.1: Characteristics of ELEMENT mother-offspring pairs with adolescent blood leukocyte DNA methylation data compared to all ELEMENT mother-infant pairs.

	ELEMENT Subset**				All ELEMENT**				
Characteristics	No.	Mean ± SD or N (%)	Min	Max	No.	Mean ± SD or N (%)	Min	Max	P-Value
Mothers									
Age at birth (yrs.)	144	26.7 ± 5.42	14.0	39.0	1458	26.1 ± 5.35	14.0	44.0	0.200
Blood lead (µg/dL)									
First Trimester	127	6.43 ± 5.16	0.90	35.8	594	5.77 ± 4.03	0.00	36.0	0.340
Second Trimester	130	5.66 ± 5.21	0.00	38.2	616	5.20 ± 3.93	0.00	38.0	0.430
Third Trimester	131	5.86 ± 4.34	0.00	34.0	575	5.54 ± 4.13	0.00	38.0	0.290
Average All Trimesters	133	5.98 ± 4.38	0.43	33.1	643	5.53 ± 3.51	0.00	33.0	0.410
Bone lead (µg/g)									
Patella	140	11.4 ± 9.45	0.11	48.0	1252	11.0 ± 8.64	0.11	68.0	0.790
Tibia	122	9.19 ± 6.88	0.12	19.0	1104	9.20 ± 7.23	0.00	44.0	0.790
Adolescent children									
Age at follow-up (yrs.)	144	14.0 ± 1.96	11.0	18.0	549	14.5 ± 2.10	10.7	18.1	0.002*
Male Sex	144	73 (51%)			546	268 (49%)			0.800
Blood Pb (µg/dL)	144	3.29 ± 4.44	0.00	41.0	404	3.09 ± 3.25	0.00	41.0	0.540
Weight (kg)	144	53.2 ± 12.2	25.9	95.0	554	54.9 ± 13.3	25.9	109.0	0.270
Height for Age Z-score	144	-0.23 ± 0.88	-2.30	-2.06	546	-0.29 ± 0.93	-2.84	3.32	0.430
BMI for Age Z-score	144	0.51 ± 1.25	-2.57	3.40	546	0.50 ± 1.25	-3.81	3.40	0.950

Note: SD – standard deviation; N – number

* p-value < 0.05 using Wilcox signed-rank test comparing entire ELEMENT cohort to the subset used in this current research.

** Participants for the current study were a subset of mother-child pairs from Cohorts 2 and 3 of ELEMENT that had archived whole blood samples for DNA methylation analysis. Here we are comparing their characteristics to those of all ELEMENT mother-child pairs from the same cohorts (2 and 3) with available data for each variable.

Table 3.2: Results from a sex-stratified sensitivity analysis of associations between Pb exposure biomarkers and DNA 5mC and 5hmC using a mixed-effects regression of repeated measures at multiple CpG sites of either 5mC or 5hmC.

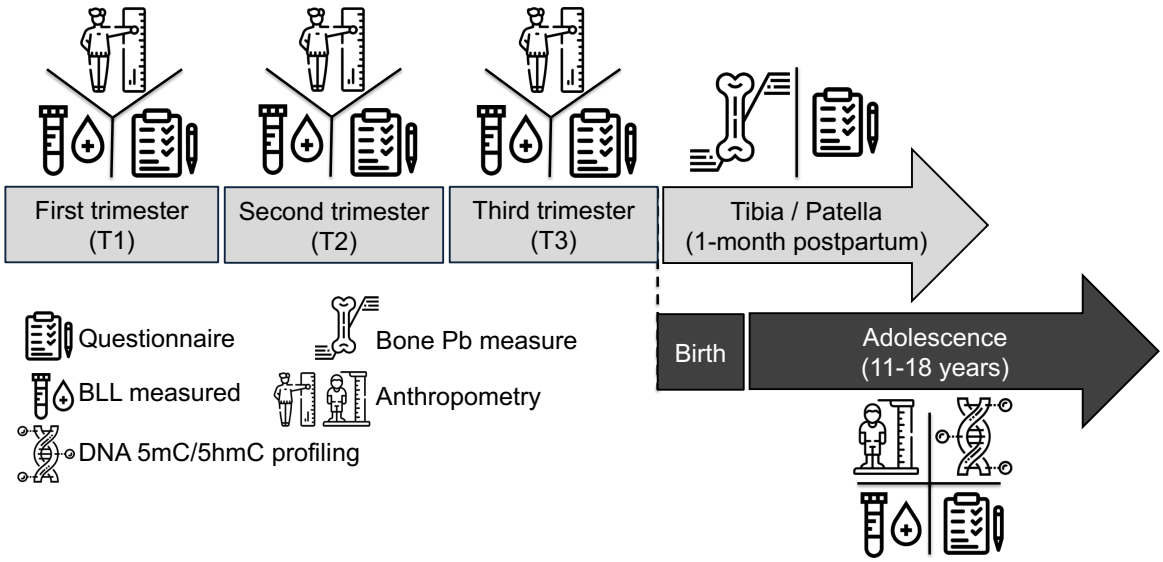
	Females			Males		
	N	**Beta (95% CI)**	***P*-value**	**N**	**Beta (95% CI)**	***P*-value**
HCN2						
T1						
5mC	61	0.41 (-0.08, 0.90)	0.101	63	0.44 (-0.22, 1.10)	0.194
5hmC	59	-0.11 (-0.34, 0.12)	0.356	61	-0.04 (-0.28, 0.20)	0.722
T2						
5mC	63	0.82 (0.25, 1.38)	0.005	64	0.02 (-0.41, 0.45)	0.922
5hmC	61	-0.35 (-0.66, -0.03)	0.031	62	0.12 (-0.17, 0.41)	0.407
T3						
5mC	64	0.84 (0.26, 1.43)	0.005	64	0.06 (-0.62, -0.03)	0.865
5hmC	62	-0.30 (-0.63, 0.03)	0.072	62	0.12 (-0.11, 0.36)	0.305
Tibia						
5mC	65	0.15 (-0.22, -0.52)	0.433	54	-0.09 (-0.40, 0.23)	0.591
5hmC	63	-0.10 (-0.30, 0.10)	0.320	52	-0.08 (-0.18, 0.02)	0.122
Patella						
5mC	67	0.17 (-0.08, 0.41)	0.183	70	0.02 (-0.19, 0.22)	0.872
5hmC	65	-0.08 (-0.21, 0.05)	0.212	68	0.04 (-0.03, 0.10)	0.323
NINJ2*						
T1						
5mC	47	-0.27 (-0.73, 0.20)	0.260	45	-0.02 (-0.60, 0.57)	0.959
5hmC	47	0.55 (0.06, 1.05)	0.029	45	0.24 (-0.32, 0.79)	0.402
T2						
5mC	46	-0.18 (-0.54, 0.18)	0.325	47	-0.11 (-0.40, 0.19)	0.477
5hmC	46	0.48 (0.10, 0.86)	0.014	47	0.07 (-0.20, 0.34)	0.617
T3						
5mC	48	-0.25 (-0.60, 0.11)	0.169	45	-0.33 (-0.81, 0.16)	0.189
5hmC	47	0.48 (0.11, 0.86)	0.012	45	0.29 (-0.17, 0.75)	0.221
Tibia						
5mC	48	0.12 (-0.56, 0.80)	0.731	39	-0.28 (-0.95, 0.40)	0.422
5hmC	48	0.10 (-0.15, 0.34)	0.449	39	0.11 (-0.09, 0.31)	0.273
Patella						
5mC	50	-0.42 (-0.87, 0.03)	0.066	51	-0.10 (-0.52, 0.33)	0.657
5hmC	50	0.20 (0.03, 0.36)	0.019	51	0.10 (-0.03, 0.24)	0.155
RAB5A						
T1						
5mC	62	0.01 (-0.02, 0.03)	0.683	64	-0.01 (-0.04, 0.02)	0.631
5hmC	62	-0.01 (-0.04, 0.04)	0.889	64	0.01 (-0.06, 0.08)	0.786
T2						
5mC	64	-0.01 (-0.04, 0.02)	0.709	65	0.01 (-0.01, 0.03)	0.281
5hmC	64	0.01 (-0.04, -0.06)	0.596	65	-0.02 (-0.05, 0.02)	0.450
T3						
5mC	65	-0.01 (-0.05, 0.02)	0.360	65	0.02 (0.00, 0.05)	0.067
5hmC	65	0.03 (-0.02, 0.08)	0.215	65	-0.03 (-0.09, 0.02)	0.241
Tibia						

	Females			Males		
	N	**Beta (95% CI)**	***P*-value**	**N**	**Beta (95% CI)**	***P*-value**
5mC	66	0.00 (-0.02, 0.02)	0.725	55	0.01 (-0.01, 0.02)	0.376
5hmC	66	0.00 (-0.03, 0.03)	0.972	55	0.01 (-0.02, 0.01)	0.529
Patella						
5mC	68	-0.01 (-0.02, 0.01)	0.444	71	0.01 (0.00, 0.01)	0.203
5hmC	68	0.01 (-0.01, 0.03)	0.390	71	-0.01 (-0.03, 0.00)	0.144
TPPP						
T1						
5mC	61	0.14 (-0.10, 0.38)	0.269	64	0.16 (-0.27, 0.60)	0.458
5hmC	61	0.01 (-0.24, 0.25)	0.965	63	0.11 (-0.46, 0.68)	0.708
T2						
5mC	63	0.03 (-0.31, 0.37)	0.865	65	0.11 (-0.15, 0.37)	0.415
5hmC	63	0.22 (-0.08, 0.52)	0.151	64	-0.07 (-0.47, 0.32)	0.717
T3				66		
5mC	64	0.17 (-0.15, 0.49)	0.297	65	0.46 (0.07, 0.85)	0.021
5hmC	64	0.14 (-0.14, 0.42)	0.335	64	-0.41 (0.07, 0.85)	0.130
Tibia						
5mC	65	-0.11 (-0.30, 0.08)	0.255	55	0.00 (-0.18, 0.18)	0.967
5hmC	65	0.06 (-0.12, 0.23)	0.517	54	0.17 (-0.09, 0.43)	0.191
Patella						
5mC	67	-0.04 (-0.16, 0.08)	0.556	71	0.16 (0.05, 0.27)	0.006
5hmC	67	0.04 (-0.08, 0.15)	0.519	70	-0.14 (-0.29, 0.02)	0.085

**NINJ2* models were also adjusted for genotype at rs34038797 (G/C).

Note: In each sex-stratified mixed-effects regression model, represented in the different rows, the outcome is percentages of 5mC or 5hmC at all quantified CpG sites treated as repeated measures, and models adjust for sex, adolescent age, adolescent BLL, and batch.

Figure 3.1: Data collection timeline from the ELEMENT birth cohort.
Maternal whole blood samples were collected during the first trimester (T1), second trimester (T2), and third trimester (T3) and analyzed for blood Pb concentrations. Maternal bone Pb was measured one month postpartum as an indicator of cumulative exposure over the course of the gestational period. Adolescent whole blood samples were collected in offspring at a follow-up visit occurring once between the ages of 11-18 years for blood Pb measures and DNA 5mC and 5hmC profiling. Covariate data including demographics and anthropometry were conducted at each stage with sample collection.



Note: BLL, Blood lead levels

Figure 3.2 Boxplot depicting DNA 5hmC percent in human whole blood detected for females and males within *HCN2* (n=137), *NINJ2* (separated by SNP (rs34038797); G/G, n=27; G/C, n=83; C/C dropped from analysis (n=34)), *RAB5A* (n=143), and *TPPP* (n=141). CpG site-specific 5hmC and 5mC averages can be found in Table 3.2A.

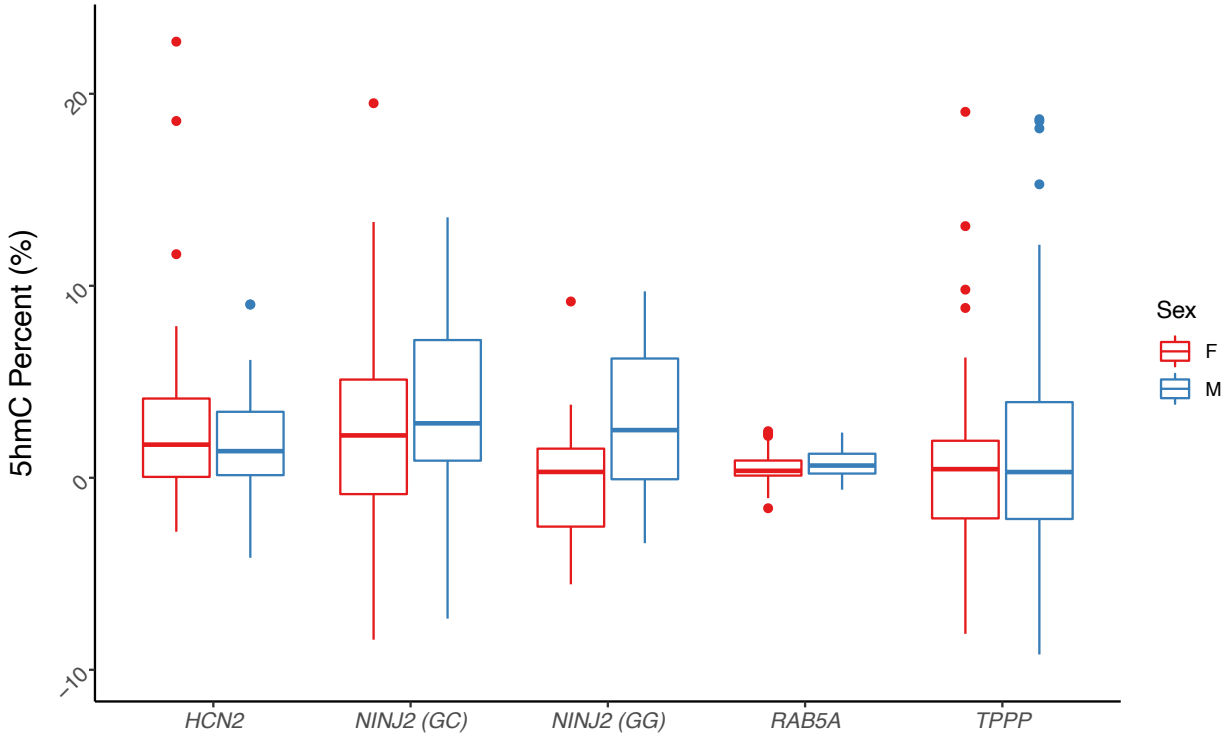
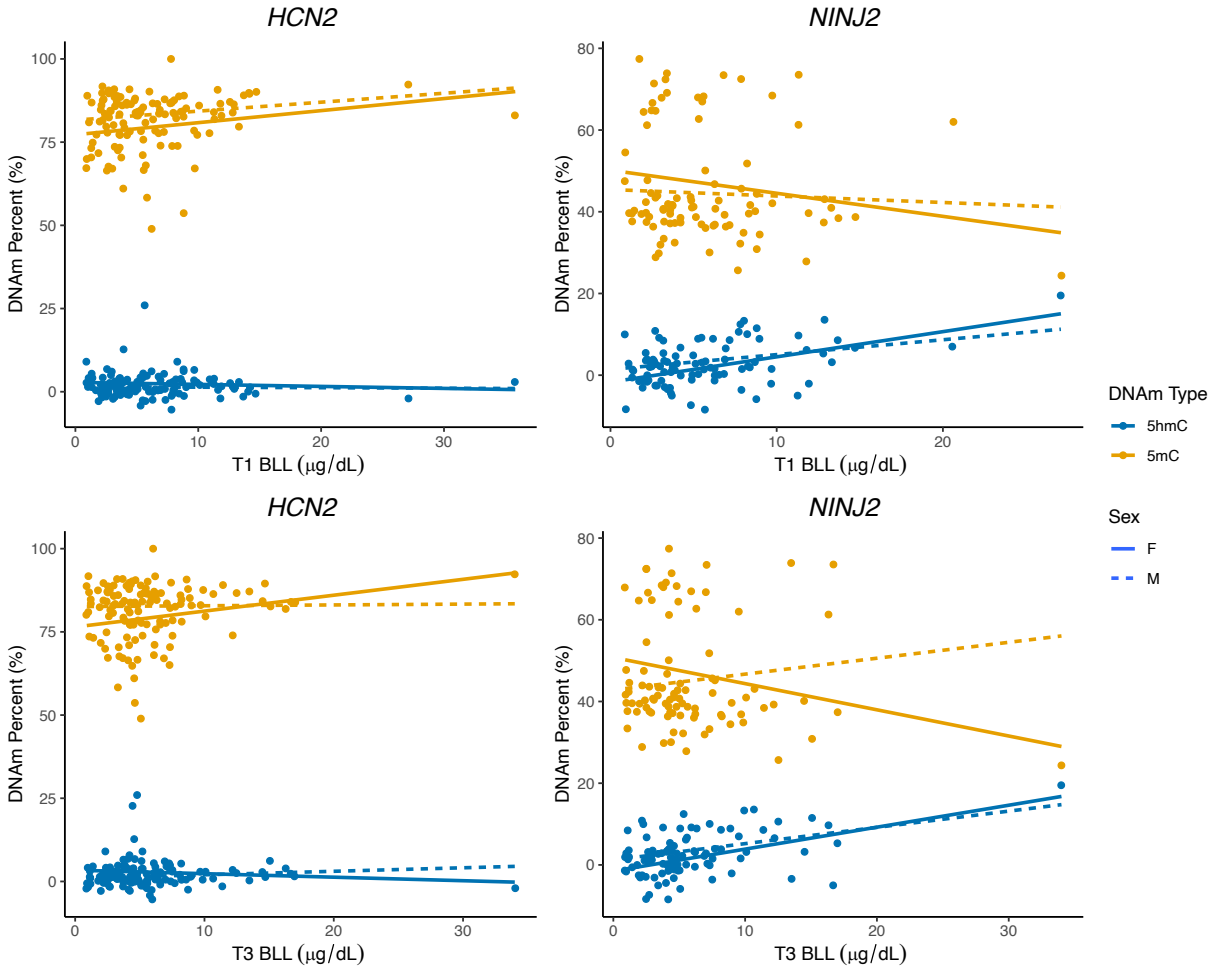
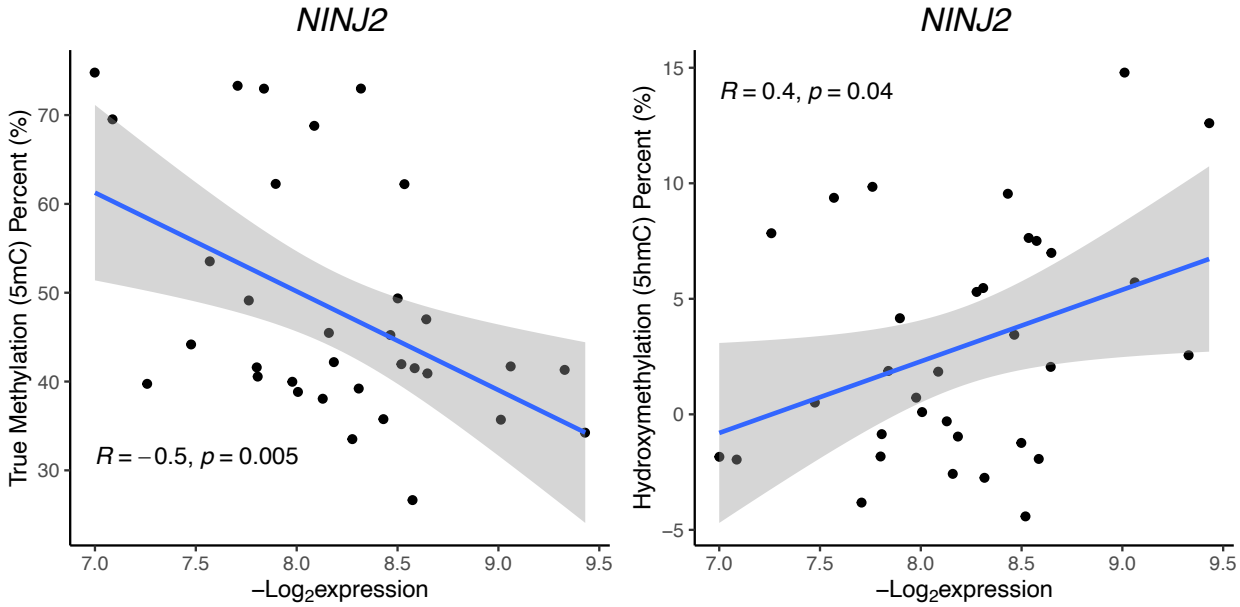


Figure 3.3: DNA 5mC (yellow) and 5hmC (blue) percent levels by T1 (top row) and T3 (bottom row) maternal Pb exposure separated by male (dashed regression line) and female (solid regression line) for loci within *HCN2* (left column) and *NINJ2* (right column). Models were adjusted for sex, adolescent age, adolescent BLL, and batch; *NINJ2* models were additionally adjusted for the SNP rs34038797.



Note: T1, First trimester; T3, third trimester; BLL, blood lead level; M, males; F, females; DNAm, DNA methylation. Models were adjusted for sex, adolescent age, adolescent BLL, and batch; *NINJ2* models were additionally adjusted for the SNP rs34038797 where C/C samples (n=34) were dropped from the analysis.

Figure 3.4: Correlation coefficients between gene expression (normalized read counts) and 5mC% (left column) and 5hmC% (right column) from pyrosequencing in *NINJ2* within a subset of individuals (n=65). Regression line indicated in blue with 95% CI in gray.



Appendix

Figure 3.1A: Correlations between gene expression and 5mC (left column) and 5hmC (right column) in *HCN2* (top row; 5mC n=68, 5hmC n=66), *TPPP* (middle row; 5mC n=63, 5hmC n=61), and *RAB5A* (bottom row; 5mC n=70, 5hmC n=70). Regression line indicated in blue with 95% confidence bands in gray.

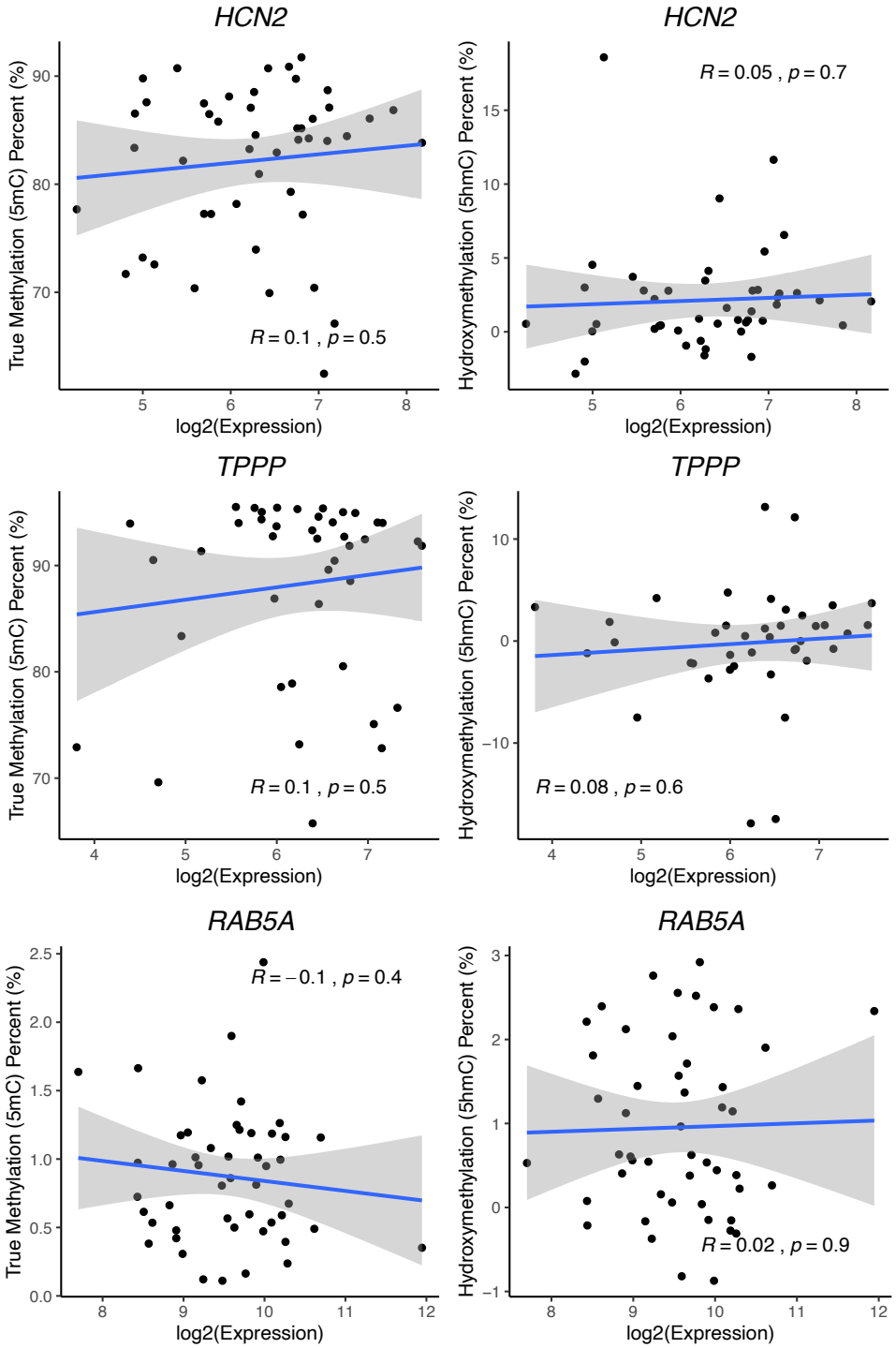


Table 3.1A: Primer sequences for oxBS-pyrosequencing.

Gene	Primer	Sequence
HCN2	Forward	5'-TTTTTTGGTTAGATTGTATTGGTAGATGT-3'
	Reverse	3'-ACTCACCTCTCCAACCCCAATTTC-5'
	Sequencing	3'-CTCTCCAACCCCAATTTCC-5'
NINJ2	Forward	5'-AGGGTTTGATTTTTATTTTTTTTAAGGT-3'
	Reverse	3'-AACCCCATTCAACTTCCTC-5'
	Sequencing	3'-CCTCCCTCAAAAACTA-5'
NINJ2	Forward	5'-TGCAAATCCCCAAAGAGG-3'
SNP Genotyping	Reverse	3'-CACCCTTGATCCTAGCACGTAC-5'
(rs34038797)	Sequencing	3'-CCAGCCCGGCCTCGT-5'
RAB5A	Forward	5'-GGAGGAGGAGAAAGGAAAGAG-3'
	Reverse	3'-AAACCCAAACTTCCTAACAACAAAACC-5'
	Sequencing	3'-AAAATAAAAATATATCTATAAC-5'
TPPP	Forward	5'-AGGGTTAGGAGAGGTAAAAATAAGA-3'
	Reverse	3'-TACCCCCTTACACCTTCTAATC-5'
	Sequencing	5'-TGTTTTTTTTGTAGAAAGTTA-3'

Table 3.2A: Average Percent (%) 5mC and 5hmC for each site, each gene, and number of samples with calculated negative 5hmC values per CpG site within *HCN2, NINJ2, RAB5A*, and *TPPP.* Bolded CpG site numbers indicate that these exact CpG sites were included on the Infinium EPIC array location of EPIC array probes.

Gene	CpG site[a]	N of 5mC	Avg 5mC[b] % ± SD	N of 5hmC	Avg 5hmC[c] % ± SD	N of Neg Samples	N of Samples with >10% 5hmC
HCN2	1	140	81.37 ± 9.53	137	3.15 ± 4.32	22	6
	2	140	83.86 ± 8.42	137	2.79 ± 3.76	28	4
	3	140	79.85 ± 7.78	137	2.19 ± 4.50	37	3
	4	140	89.49 ± 6.80	137	1.79 ± 2.83	26	2
	5	**140**	**80.86 ± 8.76**	**137**	**1.46 ± 4.58**	42	4
	6	140	72.75 ± 7.80	137	0.17 ± 5.14	61	8
	Average	140	81.31 ± 9.63	137	2.03 ± 4.31	31	3
NINJ2[a] **SNP=GG**	1	27	61.87 ± 5.17	27	5.37 ± 5.75	4	5
	2	27	74.08 ± 4.80	27	1.11 ± 4.82	10	0
	3	27	75. 92 ± 5.14	27	1.56 ± 7.21	10	3
	4	27	72.34 ± 4.77	27	0.94 ± 4.33	11	1
	5	**27**	**73.27 ± 4.68**	**27**	**-0.47 ± 4.84**	14	0
	7	**27**	**63.63 ± 4.84**	**27**	**2.64 ± 5.71**	9	3
	8	**27**	**50.24 ± 4.38**	**27**	**-0.85 ± 6.15**	16	3
	Average	27	67.34 ± 9.83	27	1.47 ± 5.86	10	0
NINJ2[d] **SNP=CG**	1	78	35.58 ± 5.32	78	5.47 ± 5.50	13	15
	2	78	42.19 ± 5.98	78	2.53 ± 5.98	21	7
	3	78	43.15 ± 6.68	78	3.14 ± 6.62	26	9
	4	78	41.25 ± 6.41	78	3.08 ± 6.19	20	14
	5	**78**	**40.96 ± 6.28**	**78**	**2.62 ± 6.53**	24	9
	7	**77**	**37.22 ± 5.87**	**77**	**2.64 ± 6.24**	26	10
	8	**78**	**30.42 ± 6.34**	**78**	**1.50 ± 6.98**	27	4
	Average	78	38.64 ± 7.39	78	3.00 ± 6.37	22	8
RAB5A	1	143	1.62 ± 0.62	143	0.74 ± 0.91	26	0
	2	143	1.40 ± 0.77	143	0.97 ± 0.94	17	0
	3	143	1.03 ± 0.77	143	0.86 ± 0.99	26	0
	4	143	1.36 ± 0.86	143	0.62 ± 1.08	39	0
	5	143	0.37 ± 0.91	143	0.33 ± 1.38	30	0
	6	143	**0.16 ± 0.45**	143	**0.40 ± 0.98**	17	0
	7	143	0.18 ± 0.90	143	0.36 ± 1.36	10	0

	8	143	0.19 ± 0.55	143	0.41 ± 1.15	16	0
	9	143	1.91 ± 1.10	143	1.46 ± 1.33	23	0
	10	143	0.32 ± 2.44	143	0.19 ± 2.51	2	0
	11	143	0.32 ± 1.00	143	0.82 ± 1.97	16	0
	Average	143	1.41 ± 1.21	143	0.64 ± 0.80	17	0
TPPP	1	141	90.30 ± 4.89	140	1.07 ± 5.27	57	6
	2	140	93.80 ± 8.41	140	1.32 ± 11.85	49	21
	3	142	94.42 ± 4.21	136	0.13 ± 5.34	57	9
	4	**142**	**90.53 ± 13.77**	**141**	**-1.15 ± 7.30**	64	18
	Average	142	92.48 ± 8.03	141	0.45 ± 8.01	55	7

Note: SD – standard deviation, Avg – average, Neg – Negative, N – Number of samples;
[a]CpG site number indicates the order of the CpG sites analyzed within the pyrosequencing region
[b]Pyrosequencing the oxidative bisulfite treated samples quantifies total percentage of 5mC.
[c]5hmC percentages were quantified by subtracting the results from the oxidative bisulfite converted samples (5mC) from standard bisulfite converted sample (5mC + 5hmC). The difference can sometimes be negative at sites with zero or low levels of methylation as a consequence of random noise.
[d]C/C samples (n=34) were dropped.

Table 3.3A: Results from three different models assessing associations between prenatal Pb biomarkers and DNA methylation. For each Pb biomarker and gene combination, the first two rows display the effect estimates for Pb and for an interaction term between the prenatal Pb biomarker and a dichotomous variable signifying whether the outcome measure is for 5mC or 5hmC; these estimates are from a mixed-effects model with repeated measures of 5hmC and 5mC for each gene as the outcome variable. The third row is a mixed-effects model of 5mC data only; in this model outcomes are repeat measures of 5mC at each CpG site within the gene and the effect estimate for the Pb biomarker is shown. The fourth row is a model of 5hmC measures only. All three models adjusted for the following covariates: sex, adolescent age, adolescent BLL, and batch. Models were identical in the sensitivity analysis with outliers of Pb exposure excluded for the 5mC only and 5hmC only models.

	All observations				Outliers excluded		
	N	**Beta[a] (95% CI)**	***P*-value**	***P*-int**	**N**	**Beta[a] (95% CI)**	***P*-value**
HCN2							
T1 Pb							
Main effect	120	0.05 (-0.12, 0.21)	0.582			NA	NA
Interaction	120	0.30 (0.16, 0.45)		<0.001		NA	NA
5mC	124	0.43 (0.07, 0.78)	0.018		122	0.58 (0.01, 1.06)	0.018
5hmC	120	-0.04 (-0.18, 0.12)	0.649		118	-0.03 (-0.23, 0.18)	0.793
T2 Pb							
Main effect	123	0.06 (-0.13, 0.24)	0.540			NA	NA
Interaction	123	0.28 (0.12, 0.45)		0.001		NA	NA
5mC	127	0.37 (0.04, 0.69)	0.026		123	0.41 (-0.16, 0.97)	0.156
5hmC	123	-0.10 (-0.29, 0.09)	0.300		119	0.01 (-0.28, 0.25)	0.931
T3 Pb							
Main effect	124	0.06 (-0.12, 0.25)	0.500			NA	NA
Interaction	124	0.32 (0.16, 0.49)		<0.001		NA	NA
5mC	128	0.50 (0.10, 0.90	0.014		127	0.39 (-0.06, 0.83)	0.091
5hmC	124	-0.06 (-0.16, 0.05)	0.547		123	0.01 (-0.20, 0.22)	0.930
Tibia							
Main effect	116	-0.03 (-0.15, 0.08)	0.553			NA	NA
Interaction	116	0.04 (-0.078, 0.15)		0.538		NA	NA
5mC	120	0.03 (-0.21, 0.26)	0.830		117	-0.02 (-0.29, 0.25)	0.878

5hmC	116	-0.06 (-0.16, 0.05)	0.318		113	-0.07 (-0.20, 0.06)	0.286
Patella							
Main effect	133	-0.01 (-0.09, 0.06)	0.724			NA	NA
Interaction	133	0.13 (0.05, 0.21)		0.001		NA	NA
5mC	137	0.10 (-0.06, 0.26)	0.211		133	0.12 (-0.08, 0.32)	0.245
5hmC	133	-0.01 (-0.09, 0.06)	0.762		129	-0.01 (-0.10, 0.00)	0.908
NINJ2[b]							
T1 Pb							
Main effect	92	0.41 (-0.02, 0.84)	0.063			NA	NA
Interaction	92	-0.82 (-1.06, -0.57)		<0.001		NA	NA
5mC	92	-0.26 (-0.59, 0.07)	0.126		90	-0.29 (-1.22, 0.64)	0.541
5hmC	92	0.49 (0.17, 0.80)	0.003		90	0.36 (0.01, 0.71)	0.042
T2 Pb							
Main effect	93	0.38 (0.24, 0.52)	<0.001			NA	NA
Interaction	93	-0.68 (-0.87, -0.49)		<0.001		NA	NA
5mC	93	-0.19 (-0.40, 0.02)	0.073		92	0.01 (-1.00, 1.01)	0.990
5hmC	93	0.27 (0.07, 0.47)	0.008		92	0.38 (0.03, 0.73)	0.034
T3 Pb							
Main effect	93	0.46 (0.29, 0.63)	<0.001			NA	NA
Interaction	93	-0.82 (-1.04, -0.60)		<0.001		NA	NA
5mC	93	-0.35 (-0.60, -0.11)	0.004		92	-0.01 (-0.79, 0.77)	0.984
5hmC	93	0.45 (0.21, 0.68)	<0.001		92	0.32 (0.03, 0.61)	0.030
Tibia							
Main effect	87	0.13 (0.01, 0.26)	0.041			NA	NA
Interaction	87	-0.28 (-0.45, -0.10)		0.002		NA	NA
5mC	87	-0.08 (-0.55, 0.39)	0.741		85	-0.17 (-0.75, 0.40)	0.558
5hmC	87	0.13 (-0.03, 0.28)	0.111		85	0.11 (-0.08, 0.30)	0.266
Patella							
Main effect	101	0.20 (0.12, 0.28)	<0.001			NA	NA
Interaction	101	-0.40 (-0.51, -0.29)		<0.001		NA	NA
5mC	101	-0.27 (-0.56, 0.03)	0.081		98	-0.36 (-0.73, 0.01)	0.056
5hmC	101	0.17 (0.07, 0.27)	<0.001		98	0.19 (0.07, 0.32)	0.003

RAB5A							
T1 Pb							
Main effect	126	-0.01 (-0.02, 0.01)	0.440			NA	NA
Interaction	126	0.01 (-0.01, 0.028)		0.421		NA	NA
5mC	126	0.01 (-0.01, 0.02)	0.454		122	-0.01 (-0.03, 0.02)	0.524
5hmC	126	-0.01 (-0.04, 0.02)	0.610		122	0.00 (-0.04, 0.05)	0.909
T2 Pb							
Main effect	129	-0.01 (-0.02, 0.01)	0.424			NA	NA
Interaction	129	0.01 (-0.01, 0.03)		0.251		NA	NA
5mC	129	0.01 (-0.01, 0.02)	0.344		124	0.00 (-0.04, 0.05)	0.817
5hmC	129	-0.01 (-0.03, 0.02)	0.625		124	0.00 (-0.04, 0.04)	0.971
T3 Pb							
Main effect	130	0.00 (-0.02, 0.02)	0.898			NA	NA
Interaction	130	0.01 (-0.02, 0.03)		0.635		NA	NA
5mC	130	0.01 (-0.01, 0.03)	0.416		129	0.01 (-0.01, 0.03)	0.414
5hmC	130	-0.01 (-0.04, 0.03)	0.851		129	-0.01 (-0.05, 0.03)	0.687
Tibia							
Main effect	122	0.01 (-0.00, 0.02)	0.193			NA	NA
Interaction	122	-0.01 (-0.03, -0.004)		0.160		NA	NA
5mC	122	0.00 (-0.01, 0.01)	0.826		119	0.00 (-0.01, 0.01)	0.945
5hmC	122	0.01 (-0.01, 0.02)	0.591		119	0.01 (-0.02, 0.03)	0.505
Patella							
Main effect	139	0.00 (-0.01, 0.01)	0.724			NA	NA
Interaction	139	0.00 (-0.01, -0.01)		0.976		NA	NA
5mC	139	0.00 (-0.01, 0.01)	0.919		134	0.00 (-0.01, 0.01)	0.606
5hmC	139	0.00 (-0.02, 0.01)	0.697		134	0.01 (-0.01, 0.02)	0.503
TPPP							
T1 Pb							
Main effect	124	-0.03 (-0.19, 0.13)	0.714			NA	NA
Interaction	124	0.14 (-0.06, 0.35)		0.174		NA	NA
5mC	125	0.11 (-0.09, 0.30)	0.281		121	0.15 (-0.13, 0.42)	0.305
5hmC	124	0.03 (-0.20, 0.26)	0.793		120	0.05 (-0.28, 0.38)	0.771

T2 Pb							
Main effect	127	0.00 (-0.17, 0.17)	0.979			NA	NA
Interaction	127	0.08 (-0.15, 0.31)		0.516		NA	NA
5mC	128	0.04 (-0.16, 0.23)	0.720		123	0.03 (-0.27, 0.33)	0.847
5hmC	127	0.06 (-0.16, 0.28)	0.599		122	0.09 (-0.27, 0.45)	0.627
T3 Pb							
Main effect	128	-0.09 (-0.27, 0.09)	0.350			NA	NA
Interaction	128	0.23 (-0.01, 0.47)		0.061		NA	NA
5mC	129	0.17 (-0.05, 0.39)	0.129		128	0.22 (-0.02, 0.45)	0.073
5hmC	128	-0.02 (-0.27, 0.22)	0.857		127	-0.12 (-0.39, 0.15)	0.372
Tibia							
Main effect	120	0.10 (-0.00, 0.20)	0.055			NA	NA
Interaction	120	-0.16 (-0.30, -0.01)		0.032		NA	NA
5mC	121	-0.06 (-0.19, 0.06)	0.342		118	0.38 (0.04, 0.71)	0.028
5hmC	120	0.11 (-0.03, 0.26)	0.133		117	0.18 (0.00, 0.36)	0.049
Patella							
Main effect	137	-0.05 (-0.12, 0.02)	0.162			NA	NA
Interaction	137	0.10 (0.01, 0.20)		0.032		NA	NA
5mC	138	0.05 (-0.04, 0.13)	0.263		133	0.21 (-0.28, 0.70)	0.404
5hmC	137	-0.04 (-0.13, 0.06)	0.432		132	-0.05 (-0.18, 0.08)	0.443

[a]Per 1 μg/dL change in maternal blood Pb at T1, T2, and T3, and 1 μg/g change in maternal bone Pb measured in tibia and patella 1-month postpartum.

[b]*NINJ2* models were controlled for genotype at rs34038797 and C/C samples (n=34) were dropped from the analysis.

Note: N, number; p-int, p-value for intercept effect estimates; T1, first trimester; T2, second trimester; T3, third trimester; In each interaction and mixed-effects regression and sensitivity analyses, the outcome is percentages of 5mC or 5hmC at all quantified CpG sites treated as repeated measures, and models adjust for sex, adolescent age, adolescent BLL, and batch.

Table 3.4A: Results from a sex-stratified sensitivity analysis of associations between Pb exposure biomarkers and DNA 5mC and 5hmC using a mixed-effects regression of repeated measures at multiple CpG sites of either 5mC or 5hmC, excluding outliers.

	Females			Males		
	N	**Beta[b] (95% CI)**	***P*-value**	**N**	**Beta[b] (95% CI)**	***P*-value**
HCN2						
T1						
5mC	59	0.83 (0.08, 1.59)	0.031	63	0.44 (-0.22, 1.10)	0.194
5hmC	57	-0.19 (-0.55, 0.18)	0.312	61	-0.04 (-0.28, 0.20)	0.722
T2						
5mC	60	0.83 (0.12, 1.54)	0.023	63	-0.01 (-0.86, 0.85)	0.988
5hmC	58	-0.30 (-0.70, 0.09)	0.134	61	0.05 (-0.24, 0.34)	0.741
T3						
5mC	63	0.74 (0.10, 1.39)	0.024	64	0.04 (-0.68, 0.75)	0.922
5hmC	61	-0.21 (-0.57, 0.15)	0.247	62	0.08 (-0.17, 0.33)	0.523
Tibia						
5mC	64	0.10 (-0.32, 0.52)	0.641	53	-0.17 (-0.54, 0.20)	0.367
5hmC	62	-0.09 (-0.32, 0.13)	0.418	51	-0.10 (-0.21, 0.02)	0.096
Patella						
5mC	65	0.21 (-0.10, 0.51)	0.187	68	0.00 (-0.29, 0.29)	0.998
5hmC	63	-0.06 (-0.22, 0.11)	0.496	66	0.02 (-0.07, 0.11)	0.656
NINJ2[a]						
T1						
5mC	46	0.03 (-1.37, 1.43)	0.97	44	-0.66 (-2.01, 0.68)	0.334
5hmC	46	0.44 (-0.07, 0.96)	0.09	44	0.27 (-0.29, 0.82)	0.349
T2						
5mC	46	0.30 (-0.88, 1.48)	0.615	44	-0.22 (-1.93, 1.48)	0.798
5hmC	46	0.36 (-0.08, 0.80)	0.108	44	0.38 (-0.22, 0.99)	0.213
T3						
5mC	47	-0.03 (-1.16, 1.10)	0.963	44	0.20 (-1.14, 1.54)	0.773
5hmC	47	0.39 (-0.02, 0.80)	0.06	44	0.13 (-0.34, 0.61)	0.591

Tibia						
5mC	47	0.33 (-0.51, 1.16)	0.446	44	-0.51 (-1.43, 0.42)	0.283
5hmC	47	0.14 (-0.17, 0.45)	0.369	44	-0.07 (-0.32, 0.18)	0.59
Patella						
5mC	49	-0.52 (-1.03, -0.02)	0.042	44	-0.32 (-0.99, 0.35)	0.347
5hmC	49	0.26 (0.08, 0.44)	0.006	44	0.00 (-0.22, 0.23)	0.978
RAB5A						
T1						
5mC	59	-0.01 (-0.06, 0.03)	0.55	63	-0.01 (-0.04, 0.02)	0.633
5hmC	59	0.01 (-0.06, 0.09)	0.717	63	0.01 (-0.04 0.02)	0.786
T2						
5mC	60	-0.02 (-0.05, 0.02)	0.427	64	0.02 (-0.01, 0.06)	0.213
5hmC	60	0.03 (-0.03, 0.09)	0.369	64	-0.02 (-0.09, 0.06)	0.679
T3						
5mC	64	-0.02 (-0.06, 0.02)	0.39	66	0.03 (0.01, 0.06)	0.014
5hmC	64	0.04 (-0.02, 0.09)	0.202	66	-0.05, -0.11, 0.01)	0.092
Tibia						
5mC	65	0.00 (-0.02, 0.02)	0.951	54	0.00 (-0.01, 0.02)	0.599
5hmC	65	-0.01 (-0.04, 0.03)	0.743	54	0.02 (-0.01, 0.05)	0.186
Patella						
5mC	65	-0.01 (-0.02, 0.01)	0.315	69	0.00 (-0.01, 0.02)	0.544
5hmC	65	0.02 (-0.01, 0.04)	0.206	69	0.00 (-0.02, 0.03)	0.95
TPPP						
T1						
5mC	58	0.18 (-0.22, 0.59)	0.376	63	0.16 (-0.28, 0.60)	0.475
5hmC	58	0.04 (-0.38, 0.46)	0.844	62	0.11 (-0.47, 0.70)	0.703
T2						
5mC	59	-0.03 (-0.42, 0.36)	0.875	64	0.28 (-0.24, 0.81)	0.291
5hmC	59	0.25 (-0.10, 0.60)	0.163	63	-0.05 (-0.74, 0.64)	0.885
T3						
5mC	63	0.16 (-0.18, 0.49)	0.36	64	0.44 (0.07, 0.81)	0.021
5hmC	63	0.11 (-0.19, 0.40)	0.484	63	-0.43 (-0.94, 0.07)	0.092

Tibia						
5mC	64	-0.02 (-0.23, 0.19)	0.859	54	-0.09 (-0.30, 0.12)	0.387
5hmC	64	0.04 (-0.16, 0.24)	0.694	53	0.31 (0.00, 0.61)	0.047
Patella						
5mC	64	-0.06 (-0.21, 0.10)	0.479	69	0.21 (0.05, 0.36)	0.008
5hmC	64	0.06 (-0.08, 0.20)	0.393	68	-0.21 (-0.41, 0.00)	0.051

Note: N, number; T1, first trimester; T2, second trimester; T3, third trimester; In each mixed-effects sensitivity analysis, the outcome is percentages of 5mC or 5hmC (separated by rows) at all quantified CpG sites treated as repeated measures stratified by sex, and models adjust for adolescent age, adolescent BLL, and batch.

[a]*NINJ2* models were controlled for genotype at rs34038797 (G/C) and C/C samples (n=34) were dropped from the analysis.

[b]Per 1 μg/dL change in maternal blood Pb at T1, T2, or T3, and 1 μg/g change in maternal bone Pb measured in tibia and patella 1-month postpartum.

Chapter 4 DNA Methylation at Birth Potentially Mediates the Association between Prenatal Lead (Pb) Exposure and Neurodevelopmental Outcomes

Abstract

Early-life lead (Pb) exposure has been linked to adverse neurodevelopmental outcomes. Recent evidence has indicated a critical role of DNA methylation in cognition, and Pb exposure has also been shown to alter DNA methylation. However, it is still unknown whether DNA methylation plays a role in the pathological mechanism of this ubiquitous metal in cognitive development. This longitudinal study investigated the associations between trimester-specific (T1, T2, T3) maternal blood Pb concentrations, gene-specific DNA methylation percentages in umbilical cord blood at birth, and infant neurodevelopmental among 85 infants from the Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) study. Infant outcomes included the Mental Development Index (MDI), Psychomotor Development Index (PDI), and Behavioral Rating Scale (BRS) of Orientation/Engagement (ORIEN) and Emotional Regulation (EMOCI), assessed at ages 12 and 24 months. DNA methylation at a locus in *CCSER1* (probe ID cg02901723) mediated the association between T2 Pb exposure on 24-month ORIEN [indirect effect estimate 4.44, 95% CI (-0.09, 10.68), P=0.06] and EMOCI percentiles [3.62 (-0.05, 8.69), P=0.05]. Cg18515027 (*GCNT1*) DNA methylation mediated the association of T1 Pb exposure with 24-month EMOCI [-4.94 (-10.6, -0.77), P=0.01], and T2 Pb with the same outcome [-3.52 (-8.09, -0.36), P=0.02], but there was a positive indirect effect estimate between T2 Pb exposure and PDI scores at 24-

months [$\beta_{Indirect}$ = 1.25 (-0.11, 3.32), P=0.09]. The indirect effect was significant for cg19703494 (*TRAPPC6A*) DNA methylation in the association between T2 Pb exposure and 24-month MDI scores [1.54 (0, 3.87), P=0.05]. A positive significant effect of cg23280166 (*VPS11*) DNA methylation on the relationship between T3 maternal BLLs and EMOCI scores at 24 months of age [$\beta_{Indirect}$ = 2.43 (-0.16, 6.38), P=0.08]. These associations provide preliminary evidence for DNA methylation at some genes as a mediator between prenatal Pb and adverse cognitive outcomes in offspring. These findings may elucidate biological pathways that could serve as potential targets for interventions to disrupt Pb toxicity.

Introduction

Lead (Pb) is an abundant element and environmental pollutant found in air, soil, and water that has been used for thousands of years due to the ease of extraction from ores, relative abundance on earth, and low cost. Examples of exposure sources common today include ingestion of contaminated water and lead-based paint in older homes. In some countries such as Mexico, Pb absorbing into food from lead-glazed ceramics used for food preparation and storage is another major source of exposure[1]. Pb is a cumulative toxicant that affects nearly all organs of which the brain is the most sensitive. Pb negatively impacts cognitive and behavioral functions, impairs learning and memory, decreases intelligence quotient (IQ), increases aggression, and may increase the risk for developing a variety of late-life neuropathological disorders [2-10]. Despite major initiatives to reduce environmental exposures, epidemiological studies demonstrate that even low levels of Pb in blood affect IQ and behavior with major impacts on functioning [11-15].

During pregnancy, Pb from past exposures stored in maternal bone is released into the bloodstream, passes through the placenta, and reaches the developing fetus [16-18]. Gestational Pb exposure has been associated with lower scores on neonatal and infant neurobehavioral tests, even when exposure levels are below 5 μg/dL in maternal blood [5] and below 2 μg/dL in cord blood [19]. In the Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) study, we previously reported that each unit of first trimester (T1) maternal blood Pb level (BLL, natural-log transformed ug/L) was associated with a decrease of 4.13 points on the Bayley Scales of Infant Development Mental Development Index (MDI) among 24-month old children [10]. Another report provided evidence that lower MDI scores as early as 6 months of age were associated with late pregnancy maternal Pb exposures below 5 μg/dL [20]. Mechanistically, the N-methyl-D-aspartate (NMDA) receptor is particularly sensitive to developmental Pb exposure due to its important role in hippocampal-dependent spatial learning and memory [21]. Studies have shown that developmental Pb exposure in rats alters NMDA subunits and signaling, which are associated with impaired hippocampal long-term potentiation representing a biological model of learning and memory [22-24]. Developmental exposures including Pb may also impact phenotype through alterations in gene regulation via epigenetic modifications such as DNA methylation [25-27].

Epigenetics is the study of mitotically heritable and potentially reversible changes in gene expression that are independent of the DNA sequence. DNA methylation (5mC) is the addition of a methyl group covalently bound to the 5'-carbon of Cytosine. In mammals, a methylated cytosine is typically adjacent to a Guanine by a phosphate, referred as a CpG site. The period of fetal development has been shown to be sensitive

to prenatal exposures, mainly because of the dramatic DNA methylation changes and reprogramming that takes places during embryogenesis [25, 26].There is current evidence for epigenetic modifications in various neurodevelopmental disorders related to deficits in language, cognition, motor skills, and other functional domains [28-31] Pb-induced oxidative stress may alter DNA methylation, which could then alter gene expression and contribute to toxicity [32]. A growing number of studies have provided evidence for gestational Pb exposure influencing offspring epigenetic programming in murine studies at candidate genes [33, 34] and epigenome-wide [35]. Further, in human studies epigenome-wide platforms have helped to identify candidate gene pathways that were potentially impacted by prenatal Pb exposure [36-39]. Among these studies, we previously utilized the Infinium MethylationEPIC to profile DNA methylation at >850,000 CpG sites and reported statistically significant associations between maternal first- and third-trimester blood Pb levels and DNA methylation at several loci [38]. Whether epigenetic mechanisms play a role in the link between prenatal Pb exposure and childhood outcomes including neurodevelopmental delays remain to be assessed in an epidemiological study.

Despite the consistent evidence for the inverse association between prenatal Pb exposure and cognitive and behavioral outcomes in childhood [5, 10, 19, 20], whether perturbation of DNA methylation is a mediator of Pb-induced neurotoxicity is largely unexplored. We hypothesized that DNA methylation differences in previously identified prenatal Pb-associated genes at birth in umbilical cord blood (UCB) mediate the association of trimester-specific maternal Pb exposure with neurodevelopmental outcomes within the first two years of life. We utilized the ELEMENT longitudinal

pregnancy cohort with rich data on trimester-specific Pb exposure and infant follow-up at 12 and 24 months of age to investigate this question. This longitudinal study builds upon our past ELEMENT study which reported an association between prenatal Pb exposure and reduced MDI score in infancy [10] and examines potential mediation of this association by DNA methylation.

Methods

Study population

The ELEMENT study consists of three longitudinal pregnancy cohorts used to investigate the influence of Pb exposure – *in utero* and in childhood – on health outcomes during sensitive periods of development. The current study includes mother-infant pairs with prenatal information from the second and third birth cohorts who were recruited at the Mexican Social Security Institute, Mexico City between 1997 and 2005. Eligibility and exclusion criteria are as previously described [40]. Maternal variables collected include age, socioeconomic status, and IQ; child variables include sex, gestational age at birth, and neurodevelopmental and anthropometric assessments at multiple follow-up visits in infancy and childhood. For the current study, 85 ELEMENT participants were selected based on availability of: prenatal blood Pb measures, archived UCB from which we could isolate DNA that passed quality control checks for DNA methylation analysis, and neurodevelopmental assessments at 12 and 24 months of age [38].

At time of recruitment, mothers were informed about the study and those who agreed to participate read and signed a letter of informed consent about the original study. Mothers also provided written, informed consent at each follow-up visit. The

Ethics Committees of the National Institutes of Public Health of Mexico, participating hospitals, and the Internal Review Board at all participating institutions including the University of Michigan, approved the research study protocol and all amendments.

Pb concentrations, DNA isolation, and DNA methylation quantification

Concentrations of Pb were measured in maternal venous blood collected during each trimester as previously described [38]. Infant venous blood was collected in trace metal-free tubes at ages 12 and 24 months and immediately refrigerated until Pb analysis using atomic absorption spectrophotometry (Model 3000; Perkin Elmer, Wellesley, MA, USA) at the Center for Environmental Health Research American British Cowdray Hospital Trace Metal Laboratory in Mexico City. For quality control, Pb analysis was repeated by the Wisconsin Laboratory of Hygiene, and precision and accuracy were demonstrated (Pearson r>0.98; mean difference <1 ug/dL between inter-laboratory replicates).

DNA was isolated from UCB, bisulfite converted, and DNA methylation was quantified utilizing the Infinium MethylationEPIC BeadChip (Illumina) [41]. Detailed description of the DNA isolation and data processing procedures is as previously described [38]. The methylation level of each CpG site was calculated as beta-values ranging from 0 (unmethylated) to 1 (methylated). CpG sites from Rygiel et. al., 2020 were selected to be tested as mediators in this analysis that fit the following criteria: CpG site was associated with a trimester-specific BLL with p<1E-5 and effect size >|0.01| and CpG site annotated to a gene related to neurodevelopment, fetal growth, or neuronal growth. The top five CpG sites meeting these criteria from each trimester of BLL were selected: Trimester 1: *RAB5A* (EPIC probe ID: cg17138393), *EXT1*

(cg00984923), *KDM6B* (cg16049335), *GCNT1* (cg18515027), *RPS29* (cg03724407); Trimester 2: *CHTF18* (cg26820233), *PRDM16* (cg12267948), *TAPBP* (cg20603557), *TRAPPC12* (cg08025337), *ICAM5* (cg10604476); Trimester 3: *GORASP2* (cg02608914), *TRAPPC6A* (cg19703494), *VPS11* (cg23280166), *MTA1* (cg20482280), *CCSER1* (cg02901723) (**Appendix Table 4.1A**).

Child neurocognitive assessment

Infant cognitive, motor, and behavioral development at 12 and 24 months was assessed by a trained personnel using the Bayley Scales of Infant Development II–Spanish version (BSID-IIS) [42] using a standardized protocol described in a previous study by our research group [43]. It is the most widely used test to identify young children with potential developmental delays. Researchers performing the test were blinded to the infant's current and past Pb exposure results. Mental Development Index (MDI), Psychomotor Development Index (PDI), and Behavioral Rating Scale (BRS) of Orientation/Engagement (ORIEN) and Emotional Regulation (EMOCI) at 12 and 24 months of age were the outcomes included in this longitudinal study (MDI-12, MDI-24, PDI-12, PDI-24, EMOCI-12, EMOCI-24, ORIEN-12, ORIEN-24). The BRS describes the child's attention, social engagement, orientation, and motivation, and may partly explain variations in individual performance based on behavior when assessing the MDI and PDI scales. MDI and PDI are classified using standardized scores. The deviation of an individual's score from that of the mean is used to classify developmental delay from this particular population: normal, mild delay, significantly delayed. Classification of developmental delay are as follows: normal (≥84), mild (≥75.4 and <84), significant delay (<75.4) for 12-month MDI and PDI scores; normal (≥85), mild (≥75.4 and <85),

significant delay (<75.4) for 24-month MDI and PDI scores. ORIEN assesses an infant's state, affect, energy, interest, exploration, and responsiveness to an examiner, along with their behavior towards those measures. EMOCI is an assessment of the infant's range of affect and emotional response to both success and failure on the assessment. Raw scores were converted to percentiles for each factor within each age group. The BRS outcomes were scored as percentiles, where lower scores reflecting more adverse behaviors. Specifically, BRS scores can be categorized as Within Normal Limits (26^{th} percentile or above), Questionable (11^{th} to 25^{th} percentile), and Non-Optimal (at or below the 10^{th} percentile). Percentiles Within Normal Limits were ≥70 for EMOCI-12, ≥72 for ORIEN-12, ≥82 for EMOCI-24, and ≥71 for ORIEN-24; Questionable were between 49-69 for EMOCI-12, 57-71 for ORIEN-12, 33-81 for EMOCI-24, 27-70 for ORIEN-24; and Non-Optimal <46 EMOCI-12, <57 for ORIEN-12, <33 for EMOCI-24, <27 for ORIEN-24 for this particular study. To our knowledge, only one other study has assessed EMOCI and ORIEN in Pb exposed infants. Their study showed exposed infants had significantly lower scores in both EMOCI and ORIEN measures when compared to unexposed infants, but no other studies have used these specific measures [44]. In our previous research with ELEMENT children (cohort 2), higher prenatal Pb exposure was associated with lower 24-month MDI scores [10]; here we expanded to include 24-month PDI, ORIEN and EMOCI, as well as 12-month MDI, PDI, ORIEN and EMOCI.

Covariates

Maternal IQ was measured using a Spanish translated version of the Information, Comprehension, Similarities, and Block Design subtests of the Wechsler Adult

Intelligence Score [45]. Additional information, such as infant weight, length, socioeconomic status, and other factors that could confound the relationship between Pb and infant development was collected. Z-scores by age and sex for length, weight and BMI were calculated by using the World Health Organization (WHO)/National Center for Health Statistics/CDC reference data [46, 47]. For each UCB sample, estimates of cell-type proportions for T lymphocytes (CD4T, CD8T), B cells, NK cells, monocytes, granulocytes, and nucleated red blood cells were performed using an established method based on UCB cell-type specific differentially methylation regions [48].

Statistical Analysis

The R Project for Statistical Computing (version 3.6.1) was used to perform all statistical analyses. Descriptive statistics were calculated for continuous measures as means and standard deviations and for categorical measures as frequencies, including sex ratios, age of mothers, maternal IQ, socioeconomic status, gestational age, infant weight, infant length/weight/BMI-for-age Z-score, infant neurodevelopmental continuous measures (MDI, PDI, EMOCI, ORIEN) and Pb biomarkers. Pb variables analyzed include maternal BLLs at each trimester (T1, T2, T3) and infant BLLs at 12 and 24 months of age, which were all treated as continuous variables. The neurodevelopmental measures at 12 and 24 months of age were considered the primary dependent variables and models as continuous scores. Maternal BLL measures at the T1, T2, and T3 were the primary exposure biomarkers. The DNA methylation data within the fifteen differentially methylated CpG sites from the UCB EPIC analysis were the mediators (**Appendix Table 4.1A**).

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Continuous variables' distributions were visually checked for normality. Current BLL and maternal trimester BLLs were natural-log transformed in all models to fit normality assumptions. We first determined associations between prenatal Pb exposure variables and covariates (e.g., maternal age, offspring sex, length-for-age z-score, weight, socioeconomic status, cell-types, etc.). We examined Spearman's rank-order correlations between continuous covariates including cell type estimates, Pb biomarkers, DNA methylation at each CpG site, and outcomes. We then chose potential confounding variables to include in final statistical mediation models that were associated with prenatal Pb, DNA methylation, and infant neurodevelopmental outcomes. Cell-types estimates were not correlated with the 15 CpG sites, exposures or outcomes; therefore, they were not included in downstream models. In final models we included potential confounders in these relationships (infant sex, maternal age at birth, maternal IQ at birth) and covariates that influence the outcome (infant BLL, infant weight and length-for-age z-scores, infant age at time of measurement).

Pairwise multivariable linear regression analyses were performed to test the outcome model (i.e., exposure → outcome; observed neurodevelopment outcomes scores at each timepoint given trimester-specific Pb exposure) and mediator models (i.e., exposure → mediator; DNA methylation per CpG-site given trimester-specific Pb exposure). This research is building upon an observed statistically significant relationship between maternal T1 BLLs and 24-month MDI score in a larger set of ELEMENT participants [10]. Our previous EWAS paper [38] reported associations between Pb and the CpG-site specific DNA methylation at the fifteen selected CpG sites. In order to explore the potential for epigenetics to be mediator and to estimate effect sizes of

mediation, this analysis was pursued even though the associations between exposure and outcome were not statistically significant at the 24-month timepoint in this subset of the ELEMENT cohort [49]. Not all ELEMENT participants included in the original publication had archived samples to isolate DNA from at birth. Thus, this pilot study has a smaller sample size and lower statistical power compared to the original study.

Mediation Models

Mediation analyses were conducted to examine whether DNA methylation at each of the CpG sites mediated the association between prenatal Pb exposure and each neurodevelopmental outcome measure [50, 51]. The analysis was performed using the algorithms previously proposed in Imai *et. al.* (2010). This incorporates the *mediate()* function within the mediation package (version 4.5.0) in R [52, 53]. In this setting, we utilize the counterfactual framework to define causal effects within mediation analysis, which relies on the following assumptions to estimate parameters:

(1) No unmeasured confounding in the relationship between exposure and outcome;

(2) No unmeasured confounding in the relationship between the mediator and outcome after adjusting for the exposure variable;

(3) No unmeasured confounding for the relationship between the exposure and mediator; and

(4) No subsequent effects that the exposure might have that may confound any mediator and outcome relationship.

The total effect (β_{Total}), direct effect (β_{Direct}), average casual mediation effect (ACME) (i.e. indirect; $\beta_{Indirect}$), and the proportion mediated were calculated using quasi-Bayesian Monte Carlo method with 2000 simulations. The β_{Direct} represents the effect of

prenatal Pb exposure on each neurodevelopmental outcome while holding DNA methylation constant; $\beta_{Indirect}$ is the estimated effect of gestational Pb exposure operating through DNA methylation; β_{Total} is β_{Direct} + $\beta_{Indirect}$; the proportion mediated is the estimated proportion of the total effect of prenatal Pb exposure on each individual outcome measure due the mediator, DNA methylation (**Figure 4.1**). Regression estimates, 95% confidence intervals, and p-values were calculated for each effect estimate. Given the pilot nature of this study and current mediation methods having less power than typical statistical methods, ACME (i.e., $\beta_{Indirect}$) with P-values<0.1 were considered statistically significant [54].

If we were to adjust for multiple comparisons, considering our analysis to include 15 independent tests (for each CpG site; the multiple exposure biomarkers and outcomes are correlated with each other and therefore are not considered completely independent tests), a p-value<0.003 would be considered statistically significant.

Since this method assumes no exposure-mediator interactions, we performed a sensitivity analysis to test for exposure-mediator interactions. We re-ran all models with T2 BLLs as the predictor and EMOCI-24 as the outcome with an exposure-mediator (DNA methylation at each CpG site) interaction term included. We also re-ran any model that had a significant ACME with the interaction term included between the exposure and mediator. There was very limited evidence for interaction (i.e., p>0.05 for effect estimate of all interaction terms except one), and as such only results from the primary model are reported here

Results

Population characteristics

Participants include 85 mother-infant dyads from ELEMENT cohorts 2 and 3 with epigenetic, Pb exposure, and outcome data. Compared with all ELEMENT cohort 2 and 3 participants that completed the BSID-II and BRS assessments and had at least one prenatal blood Pb measure, the subset including in this study did not significantly differ on maternal age at birth, trimester BLLs, IQ, socioeconomic status, or offspring sex, weight, weight/length/BMI-for-Z-score, 12-month BLL,12-month neurocognitive/behavioral outcomes, and 24-month neurocognitive outcomes; BLLs and BRS measures at 24 months were significantly higher in the subset (**Table 4.1**).

Among the 85 mothers included in this analysis, the mean age at delivery was 26.4 (standard deviation (SD)=4.8) years (**Table 4.1**). About 66% of participants were either low-middle or middle socioeconomic status. Concentrations of Pb were measured in maternal blood during the first trimester (T1) with a geometric mean of 5.27 (geometric standard deviation (GSD)=1.93) μg/dL, second trimester (T2) geometric mean of 4.74 (GSD=1.96) μg/dL, and third trimester (T3) geometric mean of 4.98 (GSD=1.93) μg/dL. Maternal BLLs between the trimesters were highly correlated ($r>0.67$) according to a Spearman's rank-order correlation test. Infant whole blood Pb geometric mean was 3.92 (GSD=1.80) at 12 months and 3.49 (GSD=1.93) μg/dL at 24-months of age, which were highly correlated ($r=0.53$) with each other and moderately correlated ($r>0.22$) with maternal trimester BLLs.

At 12 months of age, the mean weight was measured at 9.35 kg (SD=1.03), whereas the 24-month infants averaged 11.90 kg (SD=1.40). Further, the length-for-age Z-score

as measured at -0.004 (SD=1.0) in 12-month children and 0.05 (SD=0.97) in 24-month infants, putting these children within the normal height range compared to children their age.

Mean maternal IQ was measured at 93.9 (SD=19.5) points. At 12-months of age, mean MDI score was 95.1 (SD=8.33) points and PDI was 90.1 (SD=8.80) points; at 24-months of age, MDI mean was 91.1 (SD=10.9) and PDI was 96.3 (SD=8.37). Mean 12-month EMOCI percentile was 80.4 (SD=22.7) and ORIEN percentile was 85.7 (SD=19.1). At 24-months of age, mean EMOCI percentile was 80.9 (SD=22.6) and ORIEN percentile was 77.9 (SD=27.3). Each neurodevelopmental score or percentile was highly correlated between individuals at 24-months of age ($r>0.49$), but only moderately correlated at 12 months ($r>0.24$).

Mediation Analysis

This study was conducted because we previously reported a significant inverse association between T1 BLL and MDI at 24-months in a larger set of ELEMENT children [10]. In this smaller subset of 85 ELEMENT children who also have DNA methylation data from birth, we also report inverse associations between prenatal Pb and MDI (24 mos.), PDI (12 and 24 mos.), EMOCI (24 mos.), and ORIEN (24 mos.). While none of these associations were significant at $p<0.05$ in this sub-cohort, we conducted pilot mediation analyses to estimate and report on effect sizes of the potential indirect effects from DNA methylation at neurologically-relevant genes. Thus, the indirect effects are of most interest to this analysis, and indirect effects with $p<0.1$ are considered statistically significant. If adjusting for multiple-comparisons, $p<0.003$ would be significant, and none of these pilot results meet that cut-off.

Exposure-outcome models showed that at the 12-month neurodevelopment timepoint, each one-unit increase in ln-transformed Pb measured at T1, T2, and T3 was associated with decreases in PDI scores 1.39, 2.01, and 1.83, respectively, but were not statistically significant (**Appendix Table 4.2A**, β_{Total}). This non-significant inverse relationship persisted at less magnitude at 24 months with the association between T2 Pb levels and PDI scores measured at -0.27 (**Appendix Table 4.3A**, β_{Total}). T2 BLLs consistently had inverse associations with all neurodevelopmental outcomes at the 24-month timepoint. Specifically, the strongest associations measured a 6.04% (P=0.14) decrease in EMOCI percentiles and a 5.20% (P=0.30) decrease in ORIEN percentiles with on one-unit change ln-transformed T2 BLLs (**Appendix Table 4.3A**, β_{Total}). For additional exposure-outcome estimates (β_{Total}) see **Appendix Tables 4.2A** (12-month outcomes) and **4.3A** (24-month outcomes).

Analyses estimating the indirect mediating effect of gene-specific DNA methylation on the association between trimester-specific BLLs and four different neurodevelopmental outcomes at two timepoints indicated ten statistically significant ACME ($\beta_{Indirect}$, P<0.1) effect estimates out of a total of 360 mediation analyses (**Figure 4.2**, **Appendix Figure 4.1A**). For each one-unit increase in ln-transformed Pb exposure during T2, there was a 0.53% (CI: 0.23, 0.83) increase in DNA methylation within a *CCSER1* CpG site, cg02901723, at birth (P<0.001; **Figure 4.3a** $\beta_{E\text{-}M}$). The association between ln-transformed T2 BLLs and EMOCI score at 24 months of age (β_{Total}) was -6.05 (-13.97, 0.11) and the β_{Direct} (conditional on cg02901723 DNA methylation as a mediator) was -9.68 (-18.37, -1.09), thus showing that the magnitude of the association between T2 BLLs on EMOCI score at 24 months of age became larger. $\beta_{Indirect}$ for the

EMOCI percent at 24 months of age increased by 3.62% (-0.05, 8.69) for each one-unit change in ln-transformed T2 BLLs that was mediated through cg02901723 methylation (P=0.05), and the estimate for proportion mediated between the exposure and outcome by cg02901723 DNA methylation was -49%. In other words, Pb exposure was associated with decreasing EMOCI score, while increasing DNA methylation at cg02901723 was associated with an increase in EMOCI score. When there is a larger magnitude of direct effect than total effect, or if the indirect effect is in the opposite direction as the direct effect, this suggests a suppressive effect by the mediator on the direct effect between exposure and outcome [55]. Similarly, cg02901723 DNA methylation mediated the effect of T2 Pb exposure on 24-month ORIEN score [$\beta_{Indirect}$ = 4.44 (-0.09, 10.68), P=0.06]. The estimated percent of the total effect of T2 Pb exposure on 24-month ORIEN percentile due to the mediator, cg02901723 DNA methylation, is -54%.

The ACME ($\beta_{Indirect}$) for cg18515027 in the association between T1 and T2 Pb exposure and EMOCI score at 24 months of age was statistically significant (P=0.01, P=0.02). Further, cg18515027 was associated with T2 Pb and PDI scores at 12 months of age. Cg18515027 DNA methylation at birth increased by 0.42% (0.096, 0.75) with each one-unit increase in ln-transformed T2 BLLs (**Figure 4.3b** β_{E-M}; P=0.012). Regressing 24-month EMOCI scores on cg18515027 DNA methylation showed an inverse association with EMOCI scores decreasing by 8.17 (-14.67, -1.65) for each one percent increase in DNA methylation (P=0.015). Cg18515027 DNA methylation mediated the effects of T1 Pb exposure on 24-month EMOCI [$\beta_{Indirect}$ = -4.94 (-10.6, -0.77), P=0.01], and T2 Pb exposure [$\beta_{Indirect}$ = -3.52 (-8.09, -0.36), P=0.02]. The percent of the Pb-EMOCI relationship mediated by cg18515027 is estimated to be 69% for T1

BLLs and is reduced to 51% for T2 BLLs. In contrast, cg18515027 had a positive indirect effect between T2 Pb exposure and PDI scores at 24-months [$\beta_{Indirect}$ = 1.25 (-0.11, 3.32), P=0.09] with -38% mediated through DNA methylation of cg18515027 located within *GCNT1*.

The indirect effect was significant for cg19703494 DNA methylation located within *TRAPPC6a* in the association between maternal T2 Pb exposure and 24-month MDI scores [$\beta_{Indirect}$ = 1.54 (0, 3.87), P=0.05] with an estimated -31% percent mediated by cg19703494. There was a significant effect of cg23280166 DNA methylation located within *VPS11* on the relationship between T3 maternal BLLs and EMOCI scores at 24 months of age [$\beta_{Indirect}$ = 2.43 (-0.16, 6.38), P=0.08] with -19% mediated. For additional mediation effect estimates (β_{Total}, $\beta_{Indirect}$, β_{Direct}, and proportion mediated) not mentioned above, see **Appendix Tables 4.2A** (12-month outcomes) and **4.3A** (24-month outcomes).

Discussion

We conducted multiple mediation analyses in a subsample of 85 mother-child participants in the longitudinal study, ELEMENT, to begin to understand the potential for DNA methylation to be a mediator between gestational Pb exposure and offspring neurodevelopment. Specifically, we examined the association between maternal BLLs at each trimester with scores for four different domains of neurodevelopment at 12 and 24 months of age and whether UCB DNA methylation at fifteen CpG sites statistically mediated these relationships (Figure 1). This study expands upon previous ELEMENT research reporting that maternal trimester-specific BLLs predicted offspring MDI and PDI scores [10] to include EMOCI and ORIEN scores, as well as to investigate DNA

methylation as a potential mediator. Using a significance cut-off of $p<0.1$ for the ACME, we report suggestive evidence for locus-specific mediation, primarily for relationships between maternal blood Pb levels and 24-month outcomes. We provide preliminary evidence for both mediating and suppressive roles of DNA methylation in these relationships, depending on the locus. The latter of which can be interpreted as DNA methylation at a given gene suppressing the effect of Pb on a neurodevelopmental outcome [55].

Across trimesters, higher T2 maternal BLLs were most consistently associated (total effect) with lower childhood cognitive abilities at 24 months of age, though most were not statistically significant, likely to due to the sample size. Statistically significant indirect (mediation) effects by DNA methylation of cg02901723, cg18515027, and cg19703494 were also observed in models of T2 BLLs with neurodevelopmental outcomes. The majority of these indirect effects were in the opposite direction of direct effects (i.e., suppressive). When added to the model, cg02901723 DNA methylation suppressed the association between prenatal Pb exposure during T2 and ORIEN and EMOCI scores at 24 months of age. Cg02901723 (in the gene *CCSER1,* alias *FAM190A*) is frequently altered in human cancers, but recent studies have verified *CCSER1* expression in the cerebellum is associated with attention deficit hyperactivity disorder [56, 57].

DNA methylation of cg18515027 mediated associations between T1 and T2 BLL and EMOCI-24 (**Figure 4.3b**). In contrast, there was a suppressive effect of cg18515027 on the relationship between T2 BLLs and PDI-12 scores. The protein product of the gene associated with cg18515027 (*GCNT1*) behaves like a cell surface

marker to indicate whether a T-cell has received Notch signals [58]. Notch signaling stimulates proliferative signaling during neurogenesis and plays an important role in regulation of embryonic development [59, 60]. DNA methylation within cg19703494 negatively mediated the association between T1 BLLs and T2 BLLs with MDI-24. The gene that this CpG site is in - *TRAPPC6a* - is part of the TRAPP complex that plays a major role in endoplasmic reticulum-Golgi transport, but little is known about 6a specifically. Interestingly, within the TRAPP family, *TRAPP9* is associated with intellectual disability as well as microcephaly and problems with speech, and *TRAPP11* is involved in movement disorders, ataxia (nervous system degeneration), intellectual disability, and muscular dystrophy [61]. Cg23280166 was a statistically significant mediator between Pb at T3 and EMOCI-24. This site is annotated to the gene *VPS11*, which encodes an essential protein of the endosomal pathway, and it is hypothesized that in neuronal cells abnormal VPS11 functioning would attenuate the degradation of plasma membrane receptors thereby contributing to progressive developmental delay [62].

During early gestation, neurogenesis occurs at an astonishing rate starting on embryonic day 42 and ending in mid-gestation [63, 64]. By week 8, neuronal proliferation and migration beings. During T2, axons form branches (i.e., dendrites) and synapses with a cortical plate where cortical circuits are then organized [65-69]. This timing is in line with inverse associations between T1 and T2 Pb exposure and neurodevelopment measures in children up to 24-months of age that have been reported by us and others [5, 10]. By the end of T2 and into T3, the human brain contains billions of neurons [69-71]. In T3, these new cells can communicate, and the brain starts to exhibit neuronal

differentiation, axonal elongation, synapse formation and myelination [63, 64, 72-74]. The timing of these developmental processes may explain why Pb exposure during late pregnancy also correlates with early-life lower cognition scores [19, 20].

Development of the brain and nervous system *in utero* is a highly complex process, in which epigenetics plays a critical role. During gestation, a global reprogramming of epigenetic modifications in the embryo occurs making it the most vulnerable period to environmental perturbations [75]. These changes can be propagated across germ layers within the developing fetus. It has been shown that during early neuronal differentiation, DNA methylation occurs at promoters to repress germ-line specific genes, while methylation loss at other promoters activates neuronal-specific genes [76-78]. How these widespread reprogramming events relate to specific pathways of fetal neurodevelopment remains a key gap in research. It is particularly challenging, both scientifically and ethically, to research fetal development, but it is essential we enhance the understanding of mechanistic processes in order to both improve infant and maternal health, particularly in analyses of environmental exposures during pregnancy. Prenatal and early postnatal Pb exposure have been shown in rodent and human studies to be associated with epigenetic changes in the offspring including in the brain [79, 80] and blood [33, 34, 38, 81, 82]. Metals' exposure, such as to Pb, causes indirect reactive oxygen species formation with thiol depletion resulting in oxidative stress, which might be a route to explain the DNA methylation changes by Pb [83-85]. Pb-induced oxidative stress could result in oxidative damage of methylated cytosines and decrease the level of methylation.

Gestational epigenomic changes would be expected to propagate across all tissues, yet it is still important to consider tissue-specificity when conducting differential methylation research. UCB was the available tissue for at-birth DNA methylation profiling, but the most relevant tissue would ideally be brain, specifically the cortex, cerebellum, or hippocampus. We utilized a publicly available database with matched blood and adult brain DNA methylation profiles to infer the level of similarity between blood and brain at the CpG sites included in our study[86]. Of the 15 CpG sites analyzed, 8 CpGs were available in the database. Of these, 5 CpGs were moderately to highly correlated between blood and the prefrontal cortex (cg14911689, cg26654770, cg01201512, cg06657917: r>0.72; cg26371957: r=0.44) and entorhinal cortex (cg14911689, cg26654770, cg01201512, cg06657917: r>0.79; cg26371957: r=0.48), and 3 CpGs with cerebellum cg14911689, cg26654770: r>0.70; cg01201512: r=0.52). UCB DNA methylation of 3 CpG-sites were not correlated with any brain regions (cg25353752, cg00002033, cg03463208). This comparison is limited since samples in the database were from adults and our study focuses on UCB and infants. Even so, correlations between blood and brain at 5 of the 8 CpGs within this study suggest they may be able to serve as biomarkers of effects in other tissues, and this should be validated in future studies.

This pilot study is one of a growing number to consider DNA methylation as a mediator in the association between prenatal exposures and childhood phenotypes. For example, one study identified a positive mediating effect of gene-specific methylation on the association between non-syndromic cleft lip and/or palate and *in utero* Pb exposure. Pb exposure mediated 9.3% of the relationship between Pb and the outcome with a

statistically significant indirect effect (odds ratio) of 1.26 (95% CI: 1.05, 1.97) [87]. Another study reported that gene-specific differential methylation explained 12-19% of the 202g lower birthweight among offspring of women who smoked during pregnancy versus non-smokers [88]. Finally, another study identified negative mediating effects of DNA methylation at 66 CpG sites on cognitive appraisal predicting high C-peptide secretion with 54 of those CpG sites being hypomethylated and 12 CpGs hypermethylated; DNA methylation explained 5.2-32.0% of the variance, depending on the site [89]. In terms of this negative (i.e., suppressive) effect, we and others are hypothesizing that DNA methylation changes in response to some exposures may serve to protect against the impacts of that exposure. For example, while changes at some genes and/or biological pathways may be part of the mechanism leading to Pb's toxicity, changes at another set of genes may mitigate some of the toxic impacts. We cannot prove causality based on the design of our study and its' pilot nature. Future studies should confirm this hypothesis.

There are several strengths within this study, including the longitudinal design with both exposure and outcome having multiple timepoint measurements allowing the assessment of windows of susceptibility during prenatal into postnatal neurodevelopment. The selection of genes was hypothesis-driven based on associations between prenatal Pb exposure and DNA methylation at birth in the same cohort in an epigenome-wide study [38]; although, this could also be a limitation since additional genes that influence cognitive development were not examined here. DNA methylation was assessed in cord blood instead of brain. Due to the sample size limited by availability of DNA methylation data, we were unable to test whether sex differences

in the exposure-outcome or exposure-mediator-outcome relationships exist. Further, the small sample size limits our statistical power; therefore, we cannot conclude that the results presented here are not by chance alone as they did not withstand correction for multiple hypothesis testing. In a previous ELEMENT study by Hu *et. al.* (2006), the association between first trimester maternal BLLs and 24-month MDI scores was statistically significant (n=146). While we did not achieve statistical significance with the smaller subset included in this present study, the effect estimates (i.e., the total effects for the T2 and T3 BLL on MDI) were similar. The generalizability of this study may be limited to populations with similar racial-ethnic and socioeconomic backgrounds. Replication with a larger and more diverse study population is needed.

Conclusions

The results of this pilot study show that DNA methylation at several genes statistically mediates the association between T2 Pb exposure and neurodevelopmental scores at 24-months of age. This effect was independent from that of postnatal Pb exposure, and the strongest evidence for mediation was for DNA methylation of cg02901723 and cg18515027. At cg02901723, increasing methylation suppressed the influence of Pb on the outcome; whereas at cg18515027, increasing methylation mediated the association. Pb remains a global environmental problem. The majority of preventative measures focus on early childhood and often ignore prenatal exposure during embryonic development as an additional critical period affecting long-term health. Our study builds upon previous research on prenatal Pb exposure and its association with decreased childhood IQ, MDI, and adverse neurobehavioral outcomes; we provide preliminary data to consider epigenetic alterations as one of the biological mechanisms

contributing to these long-term effects. As designed, our study cannot prove causality, and we suggest future research on this topic that includes the genes that had evidence for mediation here (*CCSER1*, *GCNT1, TRAPP6A, and VPS11*). Identifying genes and their corresponding biological pathways involved in Pb's neurotoxic effects is an important step in developing interventions to disrupt or reverse toxicity.

Acknowledgements

I would like to thank my coauthors for their valuable input and feedback on this published manuscript: Dana C Dolinoy, Kelly M Bakulski, Max T. Aung, Wei Perng, Tamara R Jones, Maritsa Solano-González, Howard Hu, Martha M Tellez-Rojo, Lourdes Schnaas, Erika Marcela, Karen E Peterson, and Jaclyn M Goodrich. The authors acknowledge the American British Cowdray Hospital in Mexico City for providing research facilities. We thank the mothers and children for participating and all the research staff. This study was made possible by U.S. Environmental Protection Agency (US EPA) grants RD834800 and RD83543601, National Institute for Environmental Health Sciences (NIEHS) grants P20 ES018171, P01 ES02284401, R01 ES007821, R01 ES014930, R01 ES013744, R01 ES021446, R24-ES-028502, 1U2C ES026553, P30 ES017885, U2CES026555, U2CES026553, and R35 ES031686, and the Center for Clinical and Translational Sciences Institute KL2-TR002534 (WP). This work was also supported by the University of Michigan (UM) Genome Science Training Grant T32 HG000040 (CR). This study was supported and partially funded by the National Institute of Public Health/Ministry of Health of Mexico.

Manuscript in Review

This chapter is a slightly modified version of a manuscript in review.

Acronyms

ACME – Average casual mediation effect
BLL – Blood lead levels
BRS – Behavioral Rating Scale
BSID-IIS – Bayley Scales of Infant Development II–Spanish version

CpG – cytosine-phosphate-guanine
ELEMENT – Early Life Exposures in Mexico to Environmental Toxicants
EMOCI – Emotional Regulation
GSD – Geometric standard deviation
MDI – Mental Development Index
NMDA – N-methyl-D-aspartate
ORIEN – Orientation/Engagement
Pb – Lead
PDI – Psychomotor Development Index
SD – Standard deviation
T1 – First trimester
T2 – Second trimester
T3 – Third trimester

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Tables and Figures

Table 4.1: Characteristics of ELEMENT mother-infant pairs. Participant Pb biomarkers, DNA methylation data, and neurocognitive and behavioral outcome measures compared with outcome measures from the rest of the participants in the same ELEMENT cohorts.

	ELEMENT subset for current study			ELEMENT Cohorts 2 and 3			
Characteristics	No.	Mean ± SD or Percent (%)	Range	No.	Mean ± SD or Percent (%)	Range	P-value
Mothers							
Age at offspring birth	85	26.4 ± 4.81	18.0 - 37.0	642	26.6 ± 5.43	14.0 - 44.0	0.56
Whole blood lead (µg/dL)							
*First Trimester**	69	5.27 ± 1.93	0.90 - 35.8	594	4.78 ± 1.89	0.00 - 35.8	0.23
*Second Trimester**	74	4.74 ± 1.96	0.80 - 38.2	616	4.23 ± 1.99	0.00 - 38.2	0.15
*Third Trimester**	76	4.98 ± 1.93	0.90 - 34.0	575	4.51 ± 1.92	0.00 - 38.1	0.13
Maternal IQ	82	93.9 ± 19.5	34.0 - 139.0	581	90.4 ± 21.6	34.0 - 182.0	0.06
Maternal SES	77			506			0.82
Lowest	12	15.6		85	16.8		
Low-Middle	31	40.3		189	37.4		
Middle	20	26.0		150	29.6		
Middle-High	9	11.7		60	11.9		
Highest	5	6.49		22	4.3		
Offspring							
Male sex (%)	85	46.0		525	49.7		0.43
Whole blood lead (µg/dL)							
*12 months**	78	3.92 ± 1.80	0.90 - 19.7	466	3.85 ± 1.88	0.00 - 20.4	0.87
*24 months**	79	3.49 ± 1.93	0.80 - 17.5	522	3.98 ± 1.84	0.80 - 36.8	0.03 +
Neurological measures							

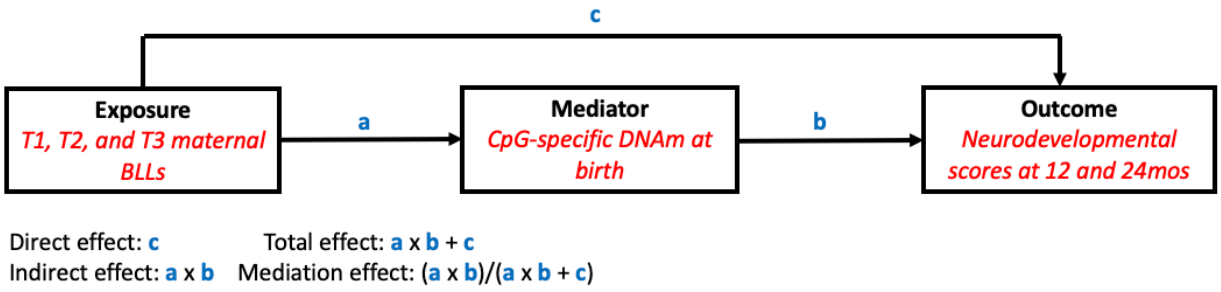
12 months							
MDI	85	95.1 ± 8.33	80.0 - 115.0	526	94.7 ± 8.95	60.0 - 116.0	0.83
PDI	85	90.1 ± 8.80	69.0 - 113.0	526	88.7 ± 8.75	50.0 - 113.0	0.34
EMOCI	84	80.4 ± 22.7	10.0 - 99.0	526	78.7 ± 22.2	6.00 - 99.0	0.99
ORIEN	84	85.7 ± 19.1	21.0 - 99.0	526	83.0 ± 20.4	9.00 - 99.0	0.34
24 months							
MDI	85	91.1 ± 10.9	72.0 - 122.0	569	86.8 ± 11.5	52.0 - 122.0	0.01[+]
PDI	85	96.3 ± 8.37	80.0 - 117.0	569	93.1 ± 10.2	61.0 - 121.0	0.01[+]
EMOCI	85	80.9 ± 22.6	13.0 - 99.0	569	77.2 ± 23.1	11.0 - 99.0	0.10
ORIEN	85	77.9 ± 27.3	10.0 - 99.0	569	74.9 ± 26.9	1.00 - 99.0	0.17
Weight (kg)							
12 months	85	9.35 ± 1.03	7.50 - 11.7	523	9.27 ± 1.10	6.30 - 14.8	0.62
24 months	85	11.9 ± 1.40	9.40 - 15.5	567	12.0 ± 1.52	8.50 - 19.5	0.58
Weight-for-age Z-score							
12 months	85	-0.04 ± 0.91	-1.93 - 2.13	523	-0.09 ± 1.00	-2.96 - 4.05	0.39
24 months	85	-0.04 ± 0.94	-1.89 - 2.32	567	0.09 ± 1.02	-3.03 - 4.27	0.78
Length-for-age Z-score							
12 months	85	-0.004 ± 1.00	-2.47 - 2.31	523	-0.08 ± 1.14	-3.50 - 4.59	0.35
24 months	85	0.05 ± 0.97	-2.07 - 2.55	567	0.28 ± 1.11	-3.00 - 5.43	0.38
BMI-for-age Z-score							
12 months	85	0.01 ± 1.01	-2.49 - 2.24	523	-0.09 ± 1.17	-4.09 - 4.39	0.35
24 months	85	0.12 ± 1.00	-2.08 - 2.62	567	0.35 ± 1.15	-3.14 - 5.60	0.33

* Geometric mean

[+] Statistically significant difference between current study population and entire ELEMENT cohort

Note: SD, Standard deviation; MDI, Mental Development Index; PDI, Psychomotor Development Index; ORIEN, Orientation/Engagement percentile; EMOCI, Emotional Regulation percentile

Figure 4.1: Diagram showing the associations tested in the mediation analyses. Direct effect refers to the association from maternal trimester-specific (T1, T2, T3) Pb concentrations in blood and neurodevelopmental scores. Indirect effect refers to the association of maternal T1, T2, or T3 Pb BLLs on neurodevelopmental outcomes at 12 and 24 months of age through CpG site-specific DNA methylation (DNAm) at birth. The total effect is the sum of the direct and indirect effects. Mediation effect is a proportion of the indirect effect compared to the total effect. Covariates included in the final model were potential confounders in these relationships (infant sex, maternal age at birth, maternal IQ at birth) and covariates that influence the outcome (infant BLL, infant weight and length-for-age z-scores, infant age at time of measurement).



Note: T1, First trimester; T2, second trimester; T3, third trimester; BLL, blood lead level; CpG, cytosine-phosphate-guanine, DNAm, DNA methylation; mos, months of age.

Figure 4.2: P-values for the average casual mediation effect (ACME), representing the influence of prenatal Pb exposure at each trimester on neurodevelopmental outcomes at 24 months of age through umbilical cord blood DNA methylation at each gene. Models control for offspring sex, current BLL, current weight, length-for-age z-score, maternal IQ and maternal age. Red dotted line is P<0.1, which is considered statistically significant.

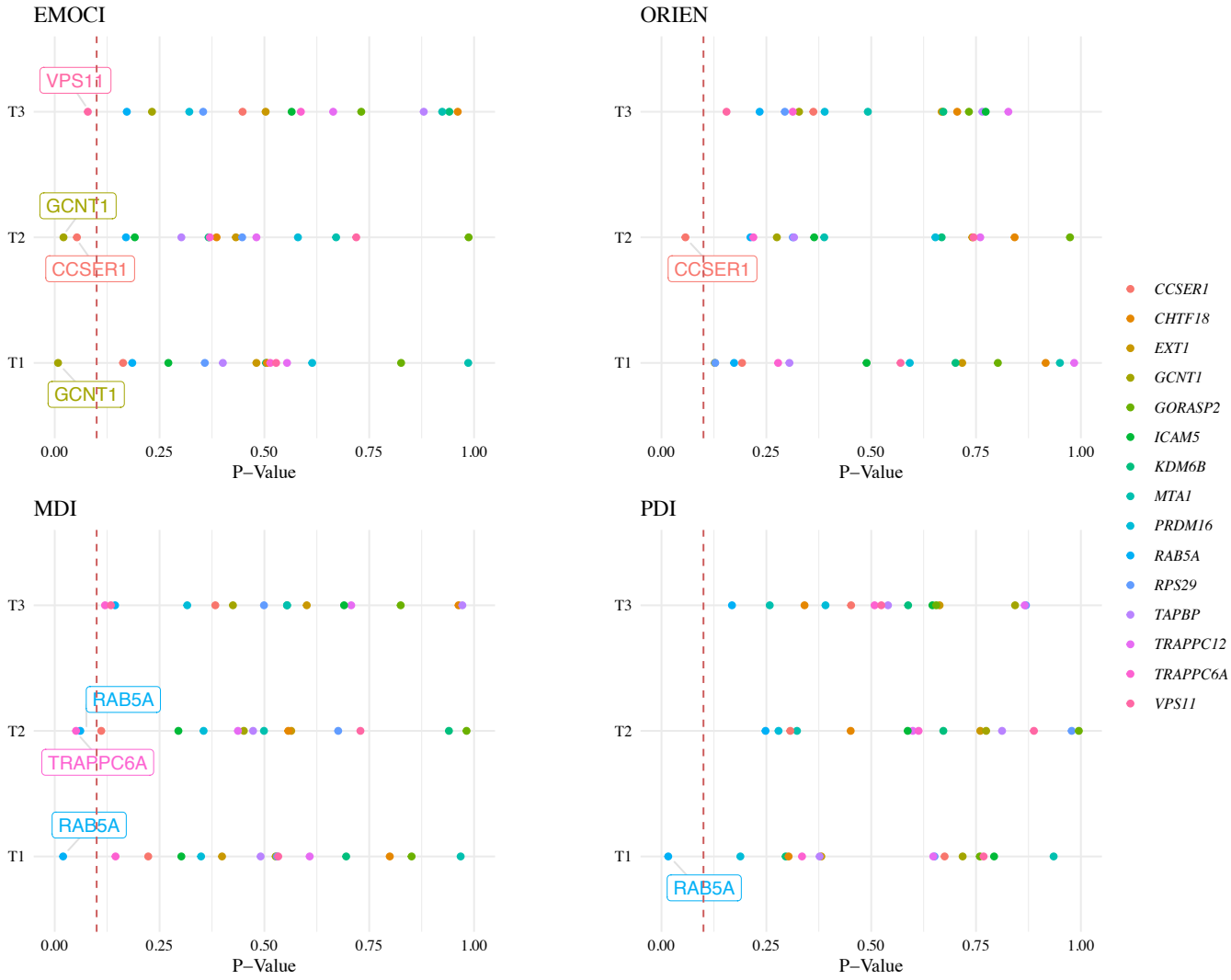
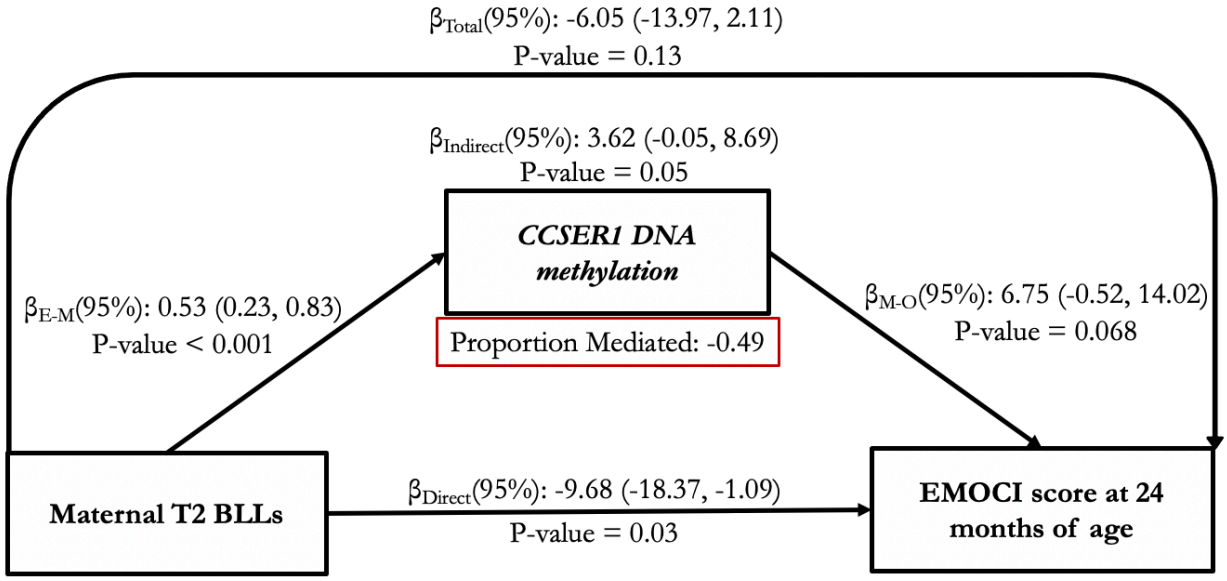
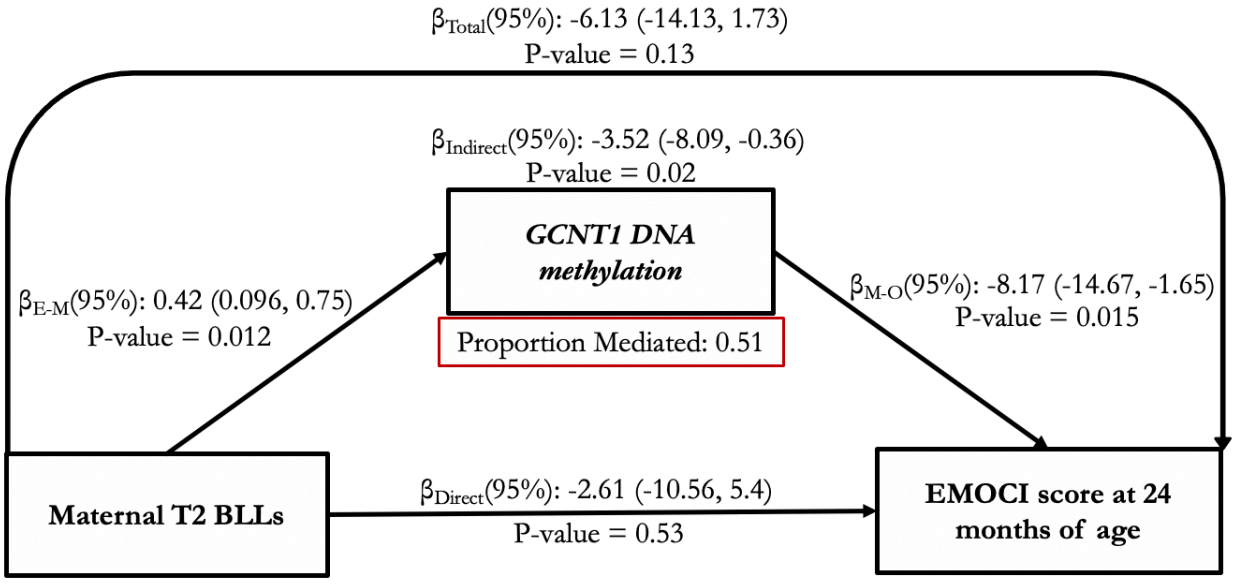


Figure 4.3: Relationship between ln-transformed T2 maternal BLLs, DNA methylation at a) cg02901723 (*CCSER1*) and b) cg18515027 (*GCNT1)*, and 24-month-old infant EMOCI scores controlling for infant sex, current BLL, current weight, length-for-age z-score, maternal IQ and maternal age. P-value<0.1 is considered statistically significant.

a)



b)



Note: T2, second trimester; BLL, blood lead level

Appendix

Table 4.1A: Gene mediators. Five differentially methylated CpG sites by prenatal Pb exposure at each trimester that were also in genes relevant to neurological function were selected from a previous epigenome-wide association study (Rygiel et al. 2020) to test as mediators in this study.

Trimester of Exposure	*EPIC CpG Probe*	*Gene Name*	*Chr.*	*Pos.*	*Beta estimate*
T1	cg17138393	*RAB5A*	chr3	19988887	-0.0221
	cg00984923	*EXT1*	chr8	119124069	-0.0235
	cg16049335	*KDM6B*	chr17	7748444	0.236
	cg18515027	*GCNT1*	chr9	79074066	0.0870
	cg03724407	*RPS29*	chr14	50053250	-0.0236
T2	cg26820233	*CHTF18*	chr16	838206	-0.0386
	cg12267948	*PRDM16*	chr1	2986566	-0.0224
	cg20603557	*TAPBP*	chr6	33281576	-0.0198
	cg08025337	*TRAPPC12*	chr2	3428370	0.154
	cg10604476	*ICAM5*	chr19	10403908	0.173
T3	cg02608914	*GORASP2*	chr2	171784720	-0.469
	cg19703494	*TRAPPC6A*	chr19	45681672	0.1733
	cg23280166	*VPS11*	chr11	118938394	-0.0496
	cg20482280	*MTA1*	chr14	105936300	-0.457
	cg02901723	*CCSER1*	chr4	91049728	0.102

*Note: Linear regression models were run for each CpG site using M-values (logit-transformed beta values) with each Pb biomarker, controlling for infant sex and cell-type proportions (granulocytes and nucleated red blood cells). P-values were obtained from the aforementioned analyses using methylation expressed as M-values. The estimates reported in this table are instead from analyses using beta-values as the outcome variable for ease of interpretation. CpG sites associated with Pb with P-values<1.0E-5 and beta estimates >|0.01| that were also in neurologically-relevant genes were selected for the current analysis. Beta estimate represents change in DNA methylation percent per one unit (ug/dL) increase in Pb. T1, first trimester; T2, second trimester; T3, third trimester; EPIC, Infinium MethylationEPIC BeadChip (Illumina); CpG, cytosine-phosphate-guanine, Chr, chromosome; Pos, base pair position

Figure 4.1A: Average casual mediation effect (ACME) p-values of the influence of prenatal Pb exposure at each trimester and potential gene mediators on adverse neurodevelopmental outcomes at 12 months of age. Models control for offspring sex, current BLL, current weight, length-for-age z-score, maternal IQ and maternal age. Red dotted line is P<0.1, which is considered statistically significant.

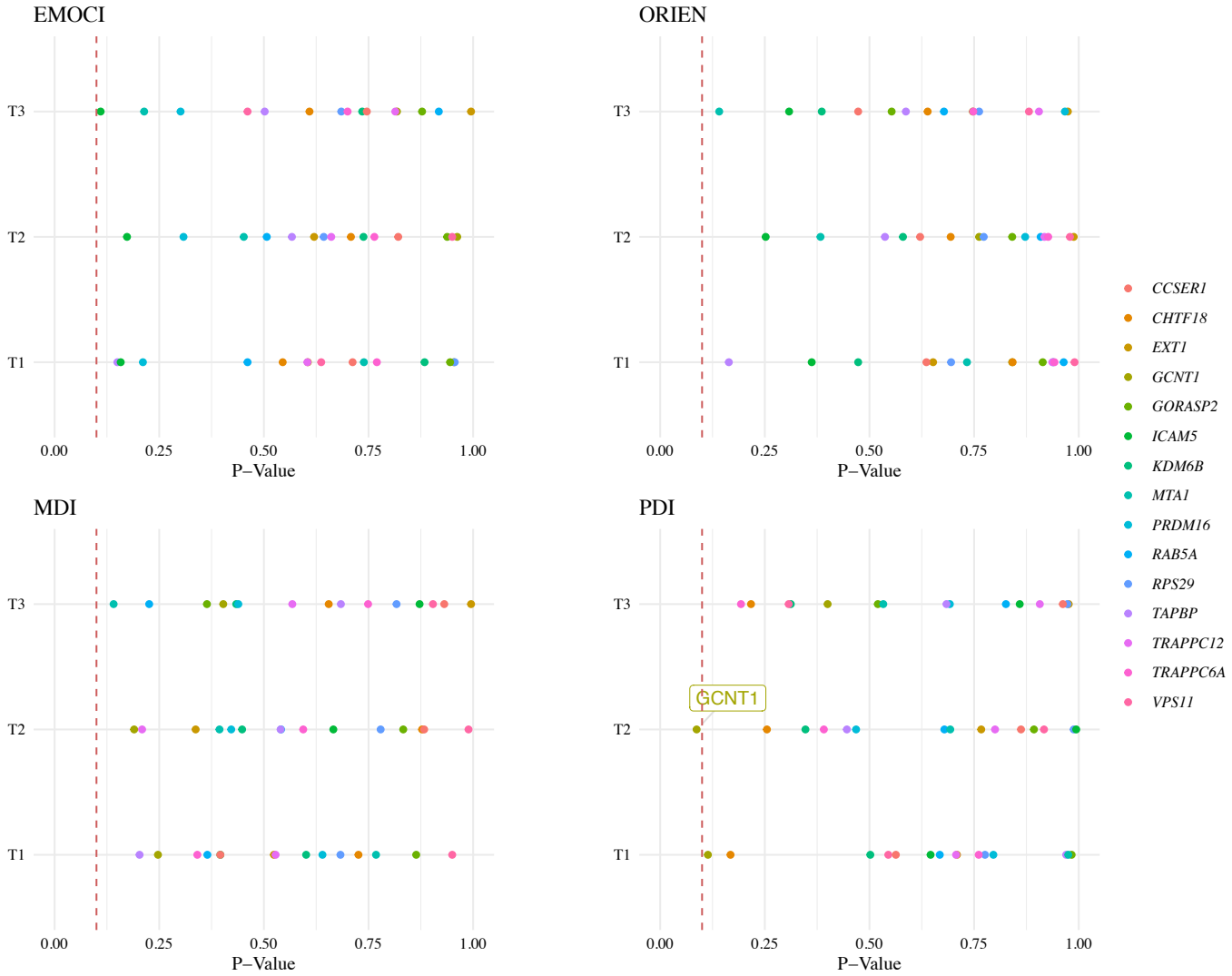


Table 4.2A: Mediation model outputs for 12-month neurodevelopmental outcomes

Exposure Timepoint	Mediator	Outcome Measure	β Total (CI)	β Total P-value	β Indirect (CI)	β Indirect P-value	β Direct (CI)	β Direct P-Value	Percent (%) Mediated
T1	*RAB5A*	EMOCI	2.95 (-6.96, 12.47)	0.56	-2.4 (-9.46, 4.12)	0.46	5.35 (-6.11, 16.41)	0.38	-24.92
		MDI	1.84 (-2.43, 5.93)	0.42	-1.21 (-4.18, 1.42)	0.37	3.04 (-1.85, 7.82)	0.24	-29.60
		ORIEN	4.5 (-3.95, 12.94)	0.34	-0.11 (-5.99, 5.82)	0.96	4.61 (-5.49, 14.35)	0.39	-1.27
		PDI	-1.4 (-5.6, 2.76)	0.52	-0.57 (-3.42, 2.18)	0.67	-0.82 (-5.73, 3.97)	0.77	12.03
	ICAM5	EMOCI	2.97 (-6.84, 12.62)	0.55	3.32 (-1.09, 9.14)	0.16	-0.35 (-11.21, 10.36)	0.97	39.06
		MDI	1.84 (-2.41, 5.96)	0.42	0.77 (-1.08, 2.94)	0.40	1.07 (-3.56, 5.68)	0.64	18.52
		ORIEN	4.52 (-4.05, 12.92)	0.32	1.85 (-2.15, 6.63)	0.36	2.67 (-6.98, 12.18)	0.57	22.46
		PDI	-1.41 (-5.5, 2.77)	0.52	-0.42 (-2.55, 1.43)	0.65	-0.99 (-5.63, 3.63)	0.67	7.86
	GORASP2	EMOCI	2.91 (-6.52, 12.73)	0.57	-0.08 (-2.19, 1.82)	0.95	2.99 (-6.46, 12.63)	0.55	0.38
		MDI	1.8 (-2.33, 6)	0.42	-0.12 (-1.87, 1.38)	0.86	1.93 (-1.93, 5.82)	0.36	2.52
		ORIEN	4.46 (-4.07, 13.08)	0.33	-0.13 (-2.82, 2.25)	0.91	4.59 (-3.49, 12.84)	0.30	0.34
		PDI	-1.41 (-5.52, 2.76)	0.51	-0.02 (-0.75, 0.66)	0.98	-1.39 (-5.5, 2.76)	0.53	0.41
	TRAPPC6A	EMOCI	2.92 (-6.63, 12.59)	0.57	-0.33 (-3.12, 1.86)	0.77	3.25 (-6.62, 13.29)	0.52	-0.81
		MDI	1.84 (-2.42, 5.96)	0.42	0.54 (-0.43, 2.14)	0.34	1.3 (-2.9, 5.57)	0.55	12.28
		ORIEN	4.5 (-3.99, 12.94)	0.34	0.09 (-2.11, 2.37)	0.94	4.41 (-4.24, 13.22)	0.34	0.51
		PDI	-1.4 (-5.58, 2.76)	0.52	0.16 (-0.9, 1.42)	0.76	-1.55 (-5.81, 2.77)	0.49	-0.90
	VPS11	EMOCI	2.96 (-6.99, 12.45)	0.56	-0.57 (-3.63, 1.44)	0.64	3.52 (-5.77, 12.93)	0.49	-1.45
		MDI	1.83 (-2.35, 6.02)	0.41	0.01 (-0.84, 0.86)	0.95	1.82 (-2.24, 5.95)	0.42	0.38
		ORIEN	4.5 (-3.96, 12.94)	0.34	-0.05 (-1.96, 1.65)	0.99	4.55 (-3.7, 12.9)	0.33	0.01
		PDI	-1.39 (-5.59, 2.81)	0.51	-0.33 (-1.82, 0.71)	0.55	-1.06 (-5, 2.96)	0.64	7.89
	MTA1	EMOCI	2.88 (-6.75, 12.7)	0.57	0.51 (-2.84, 3.94)	0.74	2.37 (-6.65, 11.42)	0.62	8.07
		MDI	1.8 (-2.31, 6.05)	0.42	0.22 (-1.4, 1.84)	0.77	1.58 (-2.22, 5.4)	0.44	9.85
		ORIEN	4.43 (-4.03, 13.1)	0.31	0.62 (-3.15, 4.38)	0.73	3.81 (-3.69, 11.35)	0.36	14.03
		PDI	-1.41 (-5.56, 2.77)	0.51	0.02 (-0.73, 0.75)	0.98	-1.42 (-5.49, 2.65)	0.51	0.19
	CCSER1	EMOCI	2.94 (-6.81, 12.57)	0.56	0.53 (-2.48, 4.01)	0.71	2.41 (-7.8, 12.71)	0.64	3.98
		MDI	1.84 (-2.42, 5.93)	0.42	0.49 (-0.61, 2.11)	0.40	1.35 (-2.96, 5.72)	0.54	10.52
		ORIEN	4.49 (-3.83, 12.97)	0.34	-0.6 (-3.71, 1.89)	0.64	5.08 (-3.84, 14.1)	0.27	-5.05
		PDI	-1.41 (-5.47, 2.79)	0.51	-0.34 (-1.85, 0.78)	0.56	-1.07 (-5.39, 3.3)	0.63	6.30
	EXT1	EMOCI	2.92 (-6.56, 12.66)	0.56	1.36 (-3.87, 7.39)	0.61	1.56 (-9.3, 12.12)	0.76	12.79
		MDI	1.82 (-2.27, 6.06)	0.41	0.73 (-1.49, 3.35)	0.52	1.09 (-3.55, 5.65)	0.65	18.78
		ORIEN	4.51 (-4.07, 12.96)	0.33	-1.03 (-6.02, 3.64)	0.65	5.54 (-3.98, 14.8)	0.28	-11.83
		PDI	-1.41 (-5.5, 2.79)	0.52	0.43 (-1.82, 3)	0.71	-1.83 (-6.47, 2.73)	0.45	-7.16
	KDM6B	EMOCI	2.93 (-6.73, 12.54)	0.57	-0.3 (-5.04, 4.16)	0.88	3.22 (-7.62, 14.02)	0.55	-3.18
		MDI	1.82 (-2.29, 6.03)	0.42	-0.46 (-2.59, 1.28)	0.60	2.29 (-2.34, 6.87)	0.33	-10.58
		ORIEN	4.48 (-3.78, 12.99)	0.33	-1.38 (-5.9, 2.26)	0.47	5.86 (-3.59, 15.25)	0.24	-15.79
		PDI	-1.41 (-5.46, 2.78)	0.52	-0.61 (-2.76, 1.11)	0.50	-0.8 (-5.4, 3.76)	0.73	13.78

		EMOCI	2.93 (-6.71, 12.52)	0.56	-0.14 (-5.39, 4.91)	0.95	3.06 (-8.11, 14.04)	0.58	-1.34
	GCNT1	MDI	1.84 (-2.43, 5.96)	0.42	1.21 (-0.77, 3.64)	0.25	0.63 (-4.1, 5.27)	0.77	32.18
		ORIEN	4.49 (-3.92, 13.01)	0.34	-0.44 (-5.16, 3.92)	0.84	4.93 (-4.86, 14.54)	0.32	-5.10
		PDI	-1.39 (-5.56, 2.77)	0.51	1.63 (-0.24, 4.21)	0.11	-3.02 (-7.67, 1.54)	0.19	-42.90
		EMOCI	2.93 (-6.7, 12.61)	0.57	0.07 (-4.12, 4.54)	0.96	2.86 (-7.44, 13.12)	0.60	0.85
	RPS29	MDI	1.82 (-2.29, 6.04)	0.41	0.34 (-1.33, 2.3)	0.68	1.49 (-2.91, 5.9)	0.52	8.20
		ORIEN	4.49 (-3.86, 13.01)	0.34	0.66 (-2.81, 4.72)	0.70	3.82 (-5.18, 12.8)	0.43	7.73
		PDI	-1.4 (-5.59, 2.79)	0.51	-0.23 (-2.12, 1.57)	0.78	-1.17 (-5.56, 3.23)	0.63	3.81
		EMOCI	2.92 (-6.56, 12.67)	0.57	0.94 (-2.11, 4.86)	0.55	1.98 (-7.8, 11.99)	0.69	7.05
	CHTF18	MDI	1.83 (-2.36, 6.01)	0.42	-0.23 (-1.84, 1.25)	0.73	2.06 (-2.17, 6.38)	0.37	-3.18
		ORIEN	4.5 (-3.98, 13.02)	0.34	-0.27 (-3.46, 2.8)	0.84	4.77 (-3.83, 13.58)	0.31	-1.91
		PDI	-1.39 (-5.59, 2.81)	0.51	-0.95 (-2.98, 0.27)	0.17	-0.44 (-4.55, 3.77)	0.86	23.93
		EMOCI	2.9 (-6.71, 12.84)	0.56	2.68 (-1.47, 8.19)	0.21	0.22 (-10.02, 10.45)	0.97	28.61
	PRDM16	MDI	1.83 (-2.4, 5.99)	0.42	-0.44 (-2.62, 1.53)	0.64	2.28 (-2.21, 6.75)	0.35	-9.49
		ORIEN	4.5 (-3.93, 12.96)	0.34	0.13 (-3.96, 4.54)	0.94	4.36 (-4.77, 13.48)	0.38	1.67
		PDI	-1.41 (-5.51, 2.76)	0.52	0.24 (-1.7, 2.43)	0.80	-1.65 (-6.12, 2.81)	0.49	-4.19
		EMOCI	2.9 (-6.77, 12.81)	0.56	2.59 (-0.89, 7.59)	0.15	0.3 (-9.68, 10.31)	0.95	28.05
	TAPBP	MDI	1.82 (-2.31, 6.09)	0.42	1.03 (-0.51, 3.17)	0.20	0.79 (-3.55, 5.15)	0.73	27.24
		ORIEN	4.47 (-4, 13.22)	0.33	2.2 (-0.83, 6.54)	0.16	2.27 (-6.49, 11.05)	0.62	28.89
		PDI	-1.4 (-5.59, 2.78)	0.51	-0.03 (-1.86, 1.87)	0.97	-1.37 (-5.78, 3.05)	0.56	-0.04
		EMOCI	2.9 (-6.75, 12.74)	0.57	-1.5 (-7.68, 4.11)	0.60	4.4 (-6.99, 15.72)	0.44	-12.98
	TRAPPC12	MDI	1.83 (-2.41, 6.01)	0.42	0.76 (-1.63, 3.34)	0.53	1.07 (-3.81, 5.93)	0.67	19.49
		ORIEN	4.48 (-3.99, 13.01)	0.33	-0.24 (-5.53, 4.91)	0.94	4.72 (-5.29, 14.67)	0.36	-2.49
		PDI	-1.41 (-5.55, 2.8)	0.51	-0.47 (-3.03, 1.93)	0.71	-0.94 (-5.82, 3.91)	0.72	9.80
		EMOCI	2.58 (-6.96, 12.13)	0.60	-1.38 (-6.37, 2.96)	0.51	3.96 (-6.3, 14.14)	0.48	-11.94
	RAB5A	MDI	0.61 (-3.46, 4.64)	0.74	-0.54 (-2.63, 1.31)	0.54	1.14 (-3.21, 5.47)	0.61	-7.99
		ORIEN	2.34 (-6.05, 10.58)	0.58	0.19 (-3.74, 4.44)	0.91	2.15 (-6.91, 11.14)	0.64	1.90
		PDI	-2.03 (-5.88, 1.75)	0.30	0.35 (-1.37, 2.35)	0.68	-2.38 (-6.56, 1.77)	0.26	-9.33
		EMOCI	2.59 (-6.98, 12.23)	0.60	3.23 (-1.18, 8.85)	0.17	-0.64 (-11.24, 9.7)	0.94	38.48
	ICAM5	MDI	0.6 (-3.44, 4.65)	0.74	0.42 (-1.54, 2.56)	0.67	0.18 (-4.36, 4.63)	0.92	7.22
		ORIEN	2.37 (-5.97, 10.72)	0.59	2.4 (-1.55, 7.19)	0.25	-0.04 (-9.41, 9.11)	0.98	30.47
T2		PDI	-2.03 (-5.9, 1.77)	0.31	0.01 (-1.99, 2)	0.99	-2.04 (-6.4, 2.23)	0.36	1.15
		EMOCI	2.55 (-6.94, 11.78)	0.59	-0.07 (-1.79, 1.36)	0.94	2.62 (-6.91, 11.78)	0.58	0.15
	GORASP2	MDI	0.58 (-3.42, 4.67)	0.77	-0.14 (-1.65, 1.11)	0.83	0.72 (-3.14, 4.44)	0.71	4.12
		ORIEN	2.31 (-6.06, 10.53)	0.60	-0.19 (-2.37, 1.65)	0.84	2.5 (-5.74, 10.43)	0.54	0.33
		PDI	-2.04 (-5.86, 1.73)	0.29	-0.05 (-0.88, 0.6)	0.89	-1.98 (-5.84, 1.73)	0.31	0.76
		EMOCI	2.55 (-6.91, 11.91)	0.59	-0.38 (-3.55, 2.3)	0.76	2.93 (-6.95, 12.81)	0.56	-1.87
	TRAPPC6A	MDI	0.61 (-3.44, 4.64)	0.74	0.27 (-0.71, 1.59)	0.59	0.34 (-3.78, 4.53)	0.85	3.36
		ORIEN	2.34 (-6, 10.59)	0.58	0.11 (-2.44, 2.71)	0.93	2.23 (-6.46, 10.92)	0.61	0.94
		PDI	-2.02 (-5.91, 1.83)	0.30	0.42 (-0.48, 1.8)	0.39	-2.44 (-6.35, 1.55)	0.21	-8.97

VPS11	EMOCI	2.58 (-6.95, 12.15)	0.60	-0.05 (-1.94, 1.65)	0.95	2.63 (-6.8, 11.9)	0.58	0.11
	MDI	0.6 (-3.42, 4.55)	0.75	0.01 (-0.67, 0.69)	0.99	0.58 (-3.43, 4.52)	0.76	0.37
	ORIEN	2.33 (-5.97, 10.49)	0.58	0.03 (-1.43, 1.45)	0.98	2.3 (-6.01, 10.47)	0.58	0.33
	PDI	-2.02 (-5.88, 1.86)	0.30	-0.04 (-1.04, 0.88)	0.92	-1.98 (-5.77, 1.75)	0.30	1.18
MTA1	EMOCI	2.52 (-6.95, 11.92)	0.61	0.87 (-1.3, 4)	0.45	1.65 (-7.58, 10.79)	0.71	8.21
	MDI	0.58 (-3.45, 4.6)	0.76	0.48 (-0.62, 1.93)	0.39	0.1 (-3.76, 3.93)	0.95	10.06
	ORIEN	2.3 (-6.08, 10.72)	0.59	1.14 (-1.47, 4.38)	0.38	1.15 (-6.74, 8.97)	0.77	16.84
	PDI	-2.04 (-5.85, 1.73)	0.30	0.14 (-0.54, 1.14)	0.69	-2.18 (-6, 1.61)	0.25	-1.34
CCSER1	EMOCI	2.57 (-6.92, 12.04)	0.59	0.51 (-4.18, 5.44)	0.82	2.06 (-8.65, 12.53)	0.69	4.52
	MDI	0.6 (-3.42, 4.6)	0.74	0.14 (-1.86, 2.2)	0.88	0.46 (-4.08, 4.9)	0.83	1.77
	ORIEN	2.33 (-5.96, 10.49)	0.58	-1 (-5.6, 2.96)	0.62	3.33 (-6.06, 12.53)	0.48	-9.06
	PDI	-2.03 (-5.88, 1.81)	0.31	0.16 (-1.75, 2.14)	0.86	-2.19 (-6.54, 2.07)	0.31	-3.50
EXT1	EMOCI	2.55 (-6.84, 11.83)	0.59	0.58 (-1.77, 3.68)	0.62	1.97 (-7.62, 11.66)	0.68	3.60
	MDI	0.59 (-3.41, 4.53)	0.76	0.5 (-0.4, 1.98)	0.34	0.09 (-3.94, 4.17)	0.95	7.28
	ORIEN	2.34 (-6, 10.6)	0.58	-0.01 (-2.34, 2.33)	0.99	2.35 (-6.1, 10.9)	0.60	0.29
	PDI	-2.03 (-5.87, 1.76)	0.30	0.14 (-0.84, 1.34)	0.77	-2.18 (-6.08, 1.77)	0.27	-2.11
KDM6B	EMOCI	2.57 (-6.94, 12.05)	0.59	0.6 (-3.35, 4.84)	0.74	1.97 (-8.34, 12.09)	0.70	4.92
	MDI	0.59 (-3.4, 4.53)	0.76	-0.63 (-2.56, 0.94)	0.45	1.22 (-3.14, 5.5)	0.58	-8.25
	ORIEN	2.33 (-5.98, 10.43)	0.58	-0.93 (-4.82, 2.37)	0.58	3.26 (-5.79, 12.14)	0.48	-8.58
	PDI	-2.04 (-5.87, 1.72)	0.29	-0.73 (-2.62, 0.75)	0.35	-1.31 (-5.48, 2.77)	0.54	21.84
GCNT1	EMOCI	2.56 (-6.98, 11.95)	0.60	-0.07 (-4.2, 3.79)	0.96	2.63 (-7.46, 12.74)	0.62	-0.52
	MDI	0.61 (-3.45, 4.64)	0.75	1.04 (-0.41, 3.09)	0.19	-0.42 (-4.64, 3.79)	0.87	21.13
	ORIEN	2.35 (-5.97, 10.72)	0.59	0.47 (-2.95, 4.03)	0.76	1.88 (-6.99, 10.76)	0.67	4.36
	PDI	-2.01 (-5.89, 1.85)	0.30	1.25 (-0.11, 3.32)	0.09	-3.26 (-7.25, 0.73)	0.10	-37.47
RPS29	EMOCI	2.59 (-6.97, 12.05)	0.60	-0.49 (-3.17, 1.37)	0.64	3.07 (-6.25, 12.36)	0.53	-1.24
	MDI	0.6 (-3.39, 4.53)	0.75	0.11 (-0.6, 1.06)	0.78	0.49 (-3.51, 4.46)	0.80	1.08
	ORIEN	2.36 (-6.05, 10.7)	0.58	-0.24 (-2.29, 1.26)	0.77	2.6 (-5.66, 10.82)	0.55	-0.54
	PDI	-2.03 (-5.88, 1.79)	0.31	-0.01 (-0.77, 0.71)	0.99	-2.01 (-5.85, 1.8)	0.30	0.15
CHTF18	EMOCI	2.55 (-6.93, 11.85)	0.59	0.65 (-2.95, 4.68)	0.71	1.9 (-8.18, 11.86)	0.71	4.89
	MDI	0.6 (-3.42, 4.61)	0.74	-0.11 (-1.76, 1.54)	0.88	0.72 (-3.56, 4.95)	0.73	-1.33
	ORIEN	2.35 (-5.99, 10.71)	0.58	-0.57 (-4.14, 2.7)	0.69	2.92 (-5.94, 11.68)	0.52	-5.04
	PDI	-2.02 (-5.9, 1.85)	0.30	-0.8 (-2.67, 0.54)	0.26	-1.22 (-5.26, 2.78)	0.56	24.44
PRDM16	EMOCI	2.54 (-6.87, 11.78)	0.60	2.15 (-1.84, 7.19)	0.31	0.38 (-9.8, 10.5)	0.92	21.22
	MDI	0.61 (-3.42, 4.67)	0.74	-0.7 (-2.83, 1.12)	0.42	1.31 (-3.04, 5.62)	0.56	-12.07
	ORIEN	2.34 (-6.05, 10.59)	0.58	0.29 (-3.57, 4.5)	0.87	2.05 (-7, 11.03)	0.65	3.06
	PDI	-2.03 (-5.89, 1.73)	0.29	0.63 (-1.02, 2.65)	0.47	-2.66 (-6.83, 1.46)	0.20	-16.73
TAPBP	EMOCI	2.55 (-6.86, 11.82)	0.60	0.91 (-2.2, 4.78)	0.57	1.64 (-8.29, 11.53)	0.74	7.02
	MDI	0.59 (-3.4, 4.52)	0.75	0.4 (-0.89, 2.03)	0.54	0.19 (-4.01, 4.37)	0.91	5.25
	ORIEN	2.33 (-5.94, 10.48)	0.58	0.86 (-1.88, 4.3)	0.54	1.47 (-7.25, 10.16)	0.73	7.80
	PDI	-2.04 (-5.89, 1.73)	0.30	0.49 (-0.71, 2.1)	0.45	-2.52 (-6.53, 1.47)	0.20	-11.29

	TRAPPC12	EMOCI	2.54 (-6.81, 11.84)	0.60	-0.54 (-3.73, 2.04)	0.66	3.08 (-6.69, 12.84)	0.53	-2.04
		MDI	0.61 (-3.49, 4.62)	0.75	0.67 (-0.39, 2.31)	0.21	-0.06 (-4.15, 4.02)	0.99	11.23
		ORIEN	2.34 (-6.02, 10.5)	0.58	0.1 (-2.42, 2.67)	0.92	2.24 (-6.38, 10.84)	0.60	1.15
		PDI	-2.03 (-5.91, 1.78)	0.31	0.12 (-0.97, 1.34)	0.80	-2.15 (-6.13, 1.83)	0.29	-1.91
T3	*RAB5A*	EMOCI	0.8 (-9.05, 10.75)	0.86	-0.17 (-3.68, 3.28)	0.92	0.97 (-8.99, 11.14)	0.84	-0.21
		MDI	2.32 (-1.99, 6.49)	0.29	-0.8 (-2.67, 0.39)	0.23	3.12 (-1.02, 7.36)	0.16	-18.09
		ORIEN	4.28 (-4.29, 12.92)	0.36	0.57 (-2.2, 3.83)	0.68	3.71 (-4.97, 12.57)	0.43	6.07
		PDI	-1.82 (-5.91, 2.31)	0.38	-0.14 (-1.59, 1.2)	0.83	-1.69 (-5.77, 2.49)	0.43	2.36
	ICAM5	EMOCI	0.84 (-9.47, 10.83)	0.86	3.62 (-0.56, 9.31)	0.11	-2.78 (-13.52, 7.61)	0.60	24.00
		MDI	2.31 (-1.88, 6.57)	0.30	0.14 (-1.77, 2.07)	0.87	2.17 (-2.5, 6.71)	0.34	2.78
		ORIEN	4.3 (-4.47, 12.95)	0.35	1.95 (-1.85, 6.51)	0.31	2.35 (-7.18, 11.58)	0.62	24.91
		PDI	-1.83 (-5.9, 2.26)	0.37	-0.16 (-2.11, 1.67)	0.86	-1.67 (-6.2, 2.74)	0.46	4.73
	GORASP2	EMOCI	0.79 (-9.05, 10.76)	0.86	0.14 (-1.82, 2.4)	0.88	0.65 (-8.98, 10.54)	0.89	0.99
		MDI	2.29 (-1.87, 6.42)	0.29	0.61 (-0.8, 2.29)	0.36	1.68 (-2.21, 5.65)	0.43	17.95
		ORIEN	4.26 (-4.23, 12.87)	0.36	0.53 (-1.08, 2.96)	0.55	3.73 (-4.6, 12.29)	0.42	4.75
		PDI	-1.84 (-5.84, 2.24)	0.37	0.27 (-0.51, 1.44)	0.52	-2.11 (-6.05, 1.92)	0.30	-3.31
	TRAPPC6A	EMOCI	0.79 (-9.03, 10.67)	0.86	-1 (-6.53, 4.01)	0.70	1.79 (-9.65, 12.77)	0.75	-4.95
		MDI	2.31 (-1.92, 6.58)	0.30	0.3 (-1.7, 2.41)	0.75	2.01 (-2.71, 6.58)	0.39	6.69
		ORIEN	4.28 (-4.32, 12.93)	0.36	-0.72 (-5.53, 3.69)	0.75	5 (-4.99, 14.6)	0.31	-8.60
		PDI	-1.81 (-5.99, 2.25)	0.38	1.24 (-0.53, 3.49)	0.19	-3.05 (-7.55, 1.31)	0.17	-34.99
	VPS11	EMOCI	0.82 (-9.16, 10.81)	0.85	-1.2 (-5.4, 2.12)	0.46	2.02 (-8.06, 12.24)	0.70	-3.53
		MDI	2.31 (-1.89, 6.52)	0.30	0.08 (-1.44, 1.69)	0.90	2.23 (-2.09, 6.59)	0.34	1.86
		ORIEN	4.28 (-4.36, 12.96)	0.36	0.21 (-2.91, 3.55)	0.88	4.07 (-4.78, 13.04)	0.40	2.34
		PDI	-1.82 (-5.98, 2.25)	0.38	-0.69 (-2.52, 0.63)	0.31	-1.13 (-5.28, 3.06)	0.59	18.35
	MTA1	EMOCI	0.76 (-8.93, 10.61)	0.86	1.71 (-0.71, 5.65)	0.21	-0.95 (-10.64, 8.9)	0.88	8.92
		MDI	2.29 (-1.81, 6.42)	0.30	0.94 (-0.21, 2.71)	0.14	1.35 (-2.7, 5.44)	0.53	26.40
		ORIEN	4.24 (-4.15, 12.78)	0.35	1.96 (-0.41, 5.65)	0.14	2.28 (-6.04, 10.75)	0.60	27.20
		PDI	-1.83 (-5.92, 2.28)	0.37	0.32 (-0.71, 1.72)	0.53	-2.15 (-6.21, 1.95)	0.30	-5.34
	CCSER1	EMOCI	0.81 (-9.14, 10.84)	0.85	0.62 (-3.54, 5.01)	0.75	0.19 (-10.49, 10.78)	0.95	2.17
		MDI	2.31 (-1.9, 6.53)	0.30	-0.07 (-1.91, 1.68)	0.93	2.37 (-2.13, 6.88)	0.30	-1.43
		ORIEN	4.27 (-4.16, 12.91)	0.36	-1.27 (-5.41, 2.1)	0.47	5.54 (-3.75, 14.75)	0.24	-14.16
		PDI	-1.83 (-5.91, 2.28)	0.38	-0.03 (-1.81, 1.67)	0.96	-1.8 (-6.17, 2.58)	0.43	1.64
	EXT1	EMOCI	0.78 (-8.92, 10.66)	0.85	-0.01 (-1.95, 1.74)	1.00	0.8 (-8.85, 10.63)	0.86	0.71
		MDI	2.3 (-1.81, 6.48)	0.30	0 (-0.95, 0.89)	1.00	2.3 (-1.78, 6.47)	0.29	0.38
		ORIEN	4.28 (-4.35, 12.97)	0.36	-0.01 (-1.42, 1.38)	0.97	4.29 (-4.18, 12.92)	0.36	0.13
		PDI	-1.83 (-5.9, 2.26)	0.37	0 (-0.66, 0.64)	0.98	-1.82 (-5.83, 2.26)	0.37	0.07
	KDM6B	EMOCI	0.81 (-9.13, 10.85)	0.85	0.58 (-3.21, 4.73)	0.74	0.22 (-10.5, 10.79)	0.94	1.86
		MDI	2.3 (-1.81, 6.5)	0.30	-0.62 (-2.5, 0.82)	0.43	2.92 (-1.62, 7.38)	0.22	-13.59
		ORIEN	4.27 (-4.18, 12.88)	0.36	-1.41 (-5.4, 1.61)	0.39	5.68 (-3.62, 14.84)	0.23	-15.32
		PDI	-1.84 (-5.82, 2.25)	0.37	-0.73 (-2.62, 0.65)	0.31	-1.1 (-5.49, 3.21)	0.62	19.89
	GCNT1	EMOCI	0.81 (-9.14, 10.86)	0.85	0.24 (-1.88, 2.73)	0.82	0.56 (-9.44, 10.62)	0.91	0.78
		MDI	2.32 (-1.99, 6.5)	0.29	0.43 (-0.48, 1.83)	0.40	1.89 (-2.3, 6.08)	0.39	9.90
		ORIEN	4.29 (-4.44, 13.02)	0.36	0.31 (-1.49, 2.58)	0.75	3.98 (-4.74, 12.76)	0.38	2.23
		PDI	-1.81 (-6, 2.24)	0.38	0.42 (-0.46, 1.79)	0.40	-2.24 (-6.3, 1.83)	0.27	-7.34
	RPS29	EMOCI	0.82 (-9.28, 10.71)	0.85	-0.43 (-3.21, 1.47)	0.69	1.26 (-8.38, 11.09)	0.80	0.23

	MDI	2.3 (-1.86, 6.51)	0.30	0.09 (-0.63, 0.94)	0.82	2.22 (-1.9, 6.43)	0.32	1.26
	ORIEN	4.3 (-4.41, 13.07)	0.36	-0.25 (-2.36, 1.21)	0.76	4.54 (-3.93, 13.19)	0.32	-0.74
	PDI	-1.82 (-5.87, 2.29)	0.38	-0.02 (-0.82, 0.68)	0.97	-1.8 (-5.81, 2.3)	0.38	0.13
CHTF18	EMOCI	0.79 (-9.02, 10.69)	0.85	0.8 (-2.33, 4.65)	0.61	-0.01 (-9.92, 10.19)	0.99	2.97
	MDI	2.31 (-1.93, 6.61)	0.30	-0.29 (-1.93, 1.13)	0.66	2.61 (-1.62, 6.94)	0.25	-5.48
	ORIEN	4.29 (-4.44, 13.08)	0.36	-0.64 (-4.01, 2.26)	0.64	4.93 (-3.73, 13.84)	0.30	-5.70
	PDI	-1.81 (-6, 2.23)	0.38	-0.82 (-2.68, 0.36)	0.22	-1 (-5.04, 3.15)	0.64	23.29
PRDM16	EMOCI	0.78 (-8.89, 10.58)	0.85	1.45 (-1.08, 5.4)	0.30	-0.67 (-10.37, 9.29)	0.93	6.13
	MDI	2.32 (-1.92, 6.55)	0.29	-0.45 (-2.07, 0.71)	0.44	2.76 (-1.38, 7.02)	0.22	-8.90
	ORIEN	4.28 (-4.36, 12.99)	0.36	0.04 (-2.68, 2.8)	0.97	4.24 (-4.33, 13.05)	0.37	0.55
	PDI	-1.83 (-5.88, 2.24)	0.37	0.23 (-0.94, 1.63)	0.69	-2.06 (-6.1, 2.09)	0.33	-3.83
TAPBP	EMOCI	0.78 (-8.89, 10.71)	0.86	0.83 (-1.45, 4.22)	0.50	-0.05 (-9.94, 9.89)	0.99	3.07
	MDI	2.3 (-1.87, 6.51)	0.30	0.21 (-0.85, 1.53)	0.68	2.1 (-2.1, 6.36)	0.36	4.25
	ORIEN	4.27 (-4.25, 12.91)	0.36	0.57 (-1.47, 3.45)	0.59	3.7 (-4.95, 12.41)	0.42	5.34
	PDI	-1.83 (-5.88, 2.25)	0.37	0.2 (-0.83, 1.49)	0.68	-2.03 (-6.11, 2.11)	0.34	-2.98
TRAPPC12	EMOCI	0.78 (-9.09, 10.79)	0.86	-0.24 (-2.59, 1.54)	0.81	1.02 (-8.97, 11.16)	0.82	0.08
	MDI	2.31 (-1.89, 6.56)	0.30	0.25 (-0.57, 1.44)	0.57	2.06 (-2.16, 6.32)	0.35	4.81
	ORIEN	4.28 (-4.26, 13.06)	0.36	0.08 (-1.67, 1.95)	0.91	4.2 (-4.54, 13.06)	0.36	0.68
	PDI	-1.83 (-5.88, 2.31)	0.38	0.04 (-0.78, 0.91)	0.91	-1.87 (-6, 2.3)	0.37	-0.03

Table 4.3A: Mediation model outputs for 24-month neurodevelopmental outcomes

Exposure Timepoint	Mediator	Outcome Measure	β Total (CI)	β Total P-value	β Indirect (CI)	β Indirect P-value	β Direct (CI)	β Direct P-Value	Percent (%) Mediated
T1	*RAB5A*	EMOCI	-5.76 (-14.7, 3.54)	0.21	-2.94 (-8.45, 1.15)	0.19	-2.82 (-12.56, 6.9)	0.59	39.07
		MDI	-0.94 (-5.76, 4.05)	0.72	-2.67 (-5.86, -0.37)	0.02	1.73 (-3.25, 6.69)	0.52	64.63
		ORIEN	-3.45 (-14.06, 7.62)	0.54	-3.58 (-10.1, 1.29)	0.17	0.13 (-11.42, 11.65)	0.98	34.83
		PDI	0.74 (-3.23, 4.85)	0.72	-2.27 (-4.92, -0.37)	0.02	3.02 (-1.07, 7.09)	0.16	-55.06
	ICAM5	EMOCI	-5.77 (-14.74, 3.57)	0.20	2.11 (-1.49, 6.86)	0.27	-7.88 (-17.58, 2.23)	0.10	-24.71
		MDI	-0.95 (-5.71, 3.96)	0.72	1.03 (-0.9, 3.5)	0.30	-1.98 (-7.15, 3.4)	0.46	-12.80
		ORIEN	-3.46 (-14.13, 7.4)	0.54	1.53 (-2.9, 6.79)	0.49	-4.99 (-16.61, 7.1)	0.41	-12.11
		PDI	0.73 (-3.21, 4.75)	0.72	0.22 (-1.5, 2.05)	0.79	0.51 (-3.8, 4.99)	0.80	3.75
	GORASP2	EMOCI	-5.8 (-15.03, 3.44)	0.22	-0.2 (-2.3, 1.48)	0.83	-5.6 (-14.62, 3.42)	0.22	1.47
		MDI	-0.97 (-5.85, 3.92)	0.72	-0.07 (-1.05, 0.71)	0.85	-0.9 (-5.71, 3.92)	0.74	0.82
		ORIEN	-3.5 (-14.46, 7.45)	0.52	-0.28 (-2.96, 1.85)	0.80	-3.22 (-13.89, 7.45)	0.57	2.24
		PDI	0.71 (-3.34, 4.67)	0.73	-0.17 (-1.54, 0.92)	0.76	0.88 (-2.96, 4.73)	0.66	1.08
	TRAPPC6A	EMOCI	-5.77 (-14.76, 3.4)	0.21	0.76 (-1.51, 3.99)	0.51	-6.54 (-15.81, 2.96)	0.17	-6.38
		MDI	-0.94 (-5.74, 4.05)	0.72	1.07 (-0.32, 3.26)	0.15	-2.01 (-6.77, 2.86)	0.42	-10.49
		ORIEN	-3.45 (-14.07, 7.62)	0.55	1.64 (-1.06, 6)	0.28	-5.08 (-15.96, 6.04)	0.36	-9.73
		PDI	0.72 (-3.33, 4.75)	0.72	-0.52 (-2.05, 0.41)	0.34	1.23 (-2.79, 5.36)	0.55	-5.03
	VPS11	EMOCI	-5.81 (-15.1, 3.26)	0.21	0.82 (-1.81, 4.04)	0.53	-6.63 (-15.42, 2.16)	0.14	-6.42
		MDI	-0.98 (-5.91, 3.86)	0.72	0.41 (-0.92, 2.06)	0.53	-1.39 (-6.07, 3.3)	0.58	-0.46
		ORIEN	-3.5 (-14.45, 7.37)	0.52	0.74 (-1.85, 4.05)	0.57	-4.24 (-14.83, 6.36)	0.44	-1.83
		PDI	0.72 (-3.29, 4.75)	0.72	0.11 (-0.62, 1.06)	0.77	0.61 (-3.37, 4.58)	0.76	1.11
	MTA1	EMOCI	-5.79 (-14.98, 3.46)	0.22	0.01 (-1.51, 1.52)	0.99	-5.8 (-14.94, 3.27)	0.21	0.08
		MDI	-0.97 (-5.86, 3.92)	0.73	0.02 (-0.84, 0.87)	0.97	-0.99 (-5.83, 3.83)	0.71	0.39
		ORIEN	-3.5 (-14.49, 7.46)	0.53	0.07 (-2.00, 2.3)	0.95	-3.57 (-14.34, 7.13)	0.52	0.46
		PDI	0.71 (-3.33, 4.74)	0.73	0.05 (-1.05, 1.17)	0.94	0.66 (-3.24, 4.53)	0.74	2.79
	CCSER1	EMOCI	-5.76 (-14.73, 3.73)	0.21	2.05 (-0.63, 6.34)	0.16	-7.81 (-17.18, 1.75)	0.09	-23.66
		MDI	-0.95 (-5.71, 4)	0.72	0.95 (-0.45, 3.15)	0.22	-1.9 (-6.91, 3.2)	0.46	-10.07
		ORIEN	-3.45 (-14.07, 7.76)	0.54	2.29 (-0.83, 7.28)	0.19	-5.74 (-16.9, 5.63)	0.30	-17.58
		PDI	0.73 (-3.22, 4.76)	0.72	0.26 (-1.02, 1.79)	0.68	0.47 (-3.73, 4.74)	0.81	3.83
	EXT1	EMOCI	-5.77 (-14.79, 3.36)	0.21	-1.27 (-5.81, 2.47)	0.48	-4.51 (-14.14, 5.1)	0.37	14.98
		MDI	-0.96 (-5.72, 3.94)	0.72	-0.8 (-3.23, 1.17)	0.40	-0.16 (-5.27, 4.93)	0.96	10.18
		ORIEN	-3.47 (-14.19, 7.41)	0.53	-0.77 (-5.85, 3.91)	0.72	-2.7 (-14.18, 8.74)	0.67	5.55
		PDI	0.73 (-3.21, 4.77)	0.72	-0.69 (-2.7, 0.92)	0.38	1.42 (-2.79, 5.61)	0.53	-12.01
	KDM6B	EMOCI	-5.77 (-14.82, 3.46)	0.21	1.34 (-2.88, 6.18)	0.50	-7.12 (-17.09, 3.21)	0.16	-15.70
		MDI	-0.97 (-5.83, 3.89)	0.72	-0.42 (-2.86, 1.79)	0.70	-0.55 (-5.86, 4.96)	0.85	4.21
		ORIEN	-3.47 (-14.14, 7.38)	0.53	0.97 (-4.16, 6.49)	0.70	-4.43 (-16.31, 7.86)	0.46	-7.64
		PDI	0.72 (-3.32, 4.76)	0.72	-0.94 (-3.13, 0.75)	0.30	1.66 (-2.67, 6.15)	0.45	-16.79
	GCNT1	EMOCI	-5.82 (-15.07, 3.06)	0.21	-4.94 (-10.6, -0.77)	0.01	-0.88 (-10.1, 8.67)	0.86	68.66
		MDI	-0.97 (-5.83, 3.93)	0.72	-0.67 (-3.05, 1.37)	0.53	-0.3 (-5.54, 5.13)	0.92	8.33
		ORIEN	-3.5 (-14.43, 7.3)	0.53	-3.38 (-9.54, 0.93)	0.13	-0.12 (-11.63, 11.78)	0.99	36.50

		PDI	0.73 (-3.22, 4.73)	0.71	0.31 (-1.48, 2.23)	0.72	0.42 (-3.92, 4.9)	0.84	5.49
	RPS29	EMOCI	-5.77 (-14.75, 3.48)	0.21	-1.64 (-6.29, 2.01)	0.36	-4.13 (-13.75, 5.45)	0.41	20.20
		MDI	-0.96 (-5.76, 3.92)	0.72	-0.59 (-2.97, 1.44)	0.53	-0.37 (-5.5, 4.74)	0.91	7.36
		ORIEN	-3.44 (-14.17, 7.86)	0.53	-3.37 (-9.43, 0.7)	0.13	-0.07 (-11.29, 11.1)	1.00	34.57
		PDI	0.73 (-3.22, 4.78)	0.71	-0.37 (-2.27, 1.34)	0.65	1.09 (-3.14, 5.31)	0.62	-6.07
	CHTF18	EMOCI	-5.8 (-15, 3.43)	0.22	0.81 (-1.45, 4.05)	0.51	-6.61 (-15.89, 2.81)	0.16	-6.88
		MDI	-0.97 (-5.8, 3.86)	0.72	0.16 (-1.22, 1.7)	0.80	-1.13 (-6.08, 3.9)	0.68	-0.07
		ORIEN	-3.48 (-14.25, 7.4)	0.54	0.13 (-3.12, 3.38)	0.92	-3.6 (-14.68, 7.64)	0.53	-0.21
		PDI	0.73 (-3.19, 4.85)	0.72	-0.58 (-2.2, 0.41)	0.30	1.31 (-2.7, 5.39)	0.54	-6.32
	PRDM16	EMOCI	-5.78 (-14.79, 3.47)	0.21	-0.96 (-5.48, 3.06)	0.61	-4.81 (-14.58, 4.82)	0.34	11.11
		MDI	-0.95 (-5.73, 3.96)	0.72	-0.93 (-3.49, 1.11)	0.35	-0.02 (-5.18, 5.06)	0.99	12.29
		ORIEN	-3.46 (-14.17, 7.47)	0.53	-1.21 (-6.6, 3.54)	0.59	-2.25 (-13.84, 9.17)	0.72	10.47
		PDI	0.73 (-3.18, 4.81)	0.72	-1.12 (-3.34, 0.45)	0.19	1.85 (-2.36, 6)	0.41	-20.51
	TAPBP	EMOCI	-5.8 (-15.04, 3.42)	0.22	1.27 (-1.51, 5.11)	0.40	-7.07 (-16.44, 2.31)	0.14	-12.63
		MDI	-0.97 (-5.85, 3.93)	0.72	0.54 (-0.96, 2.5)	0.49	-1.51 (-6.5, 3.49)	0.56	-3.71
		ORIEN	-3.5 (-14.48, 7.47)	0.53	1.78 (-1.41, 6.42)	0.31	-5.27 (-16.36, 5.82)	0.36	-12.69
		PDI	0.73 (-3.2, 4.78)	0.72	-0.55 (-2.27, 0.7)	0.38	1.28 (-2.82, 5.39)	0.56	-7.40
	TRAPPC12	EMOCI	-5.8 (-14.89, 3.43)	0.22	-1.06 (-5.48, 2.68)	0.55	-4.74 (-14.45, 4.87)	0.34	11.46
		MDI	-0.96 (-5.74, 3.94)	0.72	0.5 (-1.45, 2.74)	0.61	-1.46 (-6.63, 3.65)	0.60	-5.91
		ORIEN	-3.49 (-14.29, 7.41)	0.54	-0.01 (-4.83, 4.68)	0.98	-3.47 (-15.04, 7.98)	0.57	0.15
		PDI	0.72 (-3.3, 4.74)	0.72	-0.37 (-2.27, 1.29)	0.65	1.09 (-3.17, 5.3)	0.61	-4.58
T2	*RAB5A*	EMOCI	-6.06 (-14.03, 1.98)	0.13	-1.95 (-5.81, 0.6)	0.17	-4.11 (-12.25, 4.08)	0.32	26.31
		MDI	-1.2 (-5.6, 3.33)	0.61	-1.42 (-3.69, 0.05)	0.06	0.22 (-4.24, 4.71)	0.91	35.68
		ORIEN	-5.23 (-15, 4.62)	0.29	-2.17 (-6.83, 1.11)	0.21	-3.06 (-13.14, 7.07)	0.56	26.86
		PDI	-0.29 (-3.79, 3.23)	0.89	-0.72 (-2.38, 0.48)	0.25	0.43 (-3.19, 4.08)	0.81	7.11
	ICAM5	EMOCI	-6.06 (-14.01, 1.94)	0.13	2.68 (-1.12, 7.46)	0.19	-8.74 (-17.65, 0.05)	0.05	-35.68
		MDI	-1.21 (-5.59, 3.2)	0.60	1.18 (-1.02, 3.77)	0.30	-2.38 (-7.37, 2.54)	0.34	-24.04
		ORIEN	-5.24 (-14.86, 4.5)	0.29	2.22 (-2.68, 7.87)	0.36	-7.46 (-18.54, 3.49)	0.17	-25.43
		PDI	-0.29 (-3.8, 3.22)	0.89	0.48 (-1.29, 2.42)	0.59	-0.78 (-4.78, 3.17)	0.70	-2.12
	GORASP2	EMOCI	-6.11 (-13.97, 1.71)	0.13	-0.01 (-1.73, 1.72)	0.99	-6.1 (-13.92, 1.52)	0.12	0.23
		MDI	-1.22 (-5.61, 3.15)	0.59	0 (-0.76, 0.75)	0.98	-1.22 (-5.61, 3.05)	0.60	0.43
		ORIEN	-5.29 (-15, 4.36)	0.29	-0.01 (-2.24, 2.19)	0.97	-5.28 (-14.9, 4.1)	0.28	0.59
		PDI	-0.31 (-3.84, 3.2)	0.88	0 (-1.07, 0.98)	1.00	-0.31 (-3.7, 3)	0.87	4.75
	TRAPPC6A	EMOCI	-6.07 (-13.86, 1.83)	0.12	1.22 (-1.45, 4.78)	0.37	-7.29 (-15.66, 1.05)	0.08	-14.69
		MDI	-1.2 (-5.6, 3.34)	0.61	1.54 (0, 3.87)	0.05	-2.73 (-7.23, 1.76)	0.21	-31.19
		ORIEN	-5.23 (-15.02, 4.61)	0.29	2.16 (-1.06, 6.9)	0.22	-7.39 (-17.62, 2.82)	0.15	-23.89
		PDI	-0.3 (-3.78, 3.18)	0.89	-0.31 (-1.76, 0.93)	0.61	0.01 (-3.72, 3.72)	0.97	0.93
	VPS11	EMOCI	-6.12 (-14.16, 1.87)	0.13	0.41 (-1.93, 2.96)	0.72	-6.53 (-14.17, 0.95)	0.09	-3.20
		MDI	-1.23 (-5.63, 3.12)	0.60	0.2 (-0.97, 1.55)	0.73	-1.44 (-5.72, 2.75)	0.52	0.42
		ORIEN	-5.29 (-15.04, 4.52)	0.28	0.39 (-1.98, 3.19)	0.74	-5.68 (-15.24, 3.67)	0.23	-1.38
		PDI	-0.3 (-3.8, 3.17)	0.89	0.05 (-0.57, 0.78)	0.89	-0.36 (-3.85, 3.06)	0.86	0.42
	MTA1	EMOCI	-6.1 (-13.99, 1.8)	0.12	0.33 (-1.2, 2.49)	0.67	-6.43 (-14.27, 1.39)	0.11	-2.12
		MDI	-1.23 (-5.6, 3.17)	0.59	0.32 (-0.5, 1.63)	0.50	-1.55 (-5.88, 2.77)	0.50	-1.93
		ORIEN	-5.29 (-15.03, 4.45)	0.29	0.98 (-1.17, 4.25)	0.39	-6.28 (-15.79, 3.21)	0.19	-7.39
		PDI	-0.31 (-3.84, 3.21)	0.89	0.47 (-0.44, 1.76)	0.32	-0.78 (-4.15, 2.58)	0.68	0.27

	CCSER1	EMOCI	-6.05 (-13.97, 2.11)	0.13	3.62 (-0.05, 8.69)	0.05	-9.68 (-18.37, -1.09)	0.03	-49.24
		MDI	-1.2 (-5.64, 3.3)	0.61	1.77 (-0.25, 4.47)	0.11	-2.97 (-7.84, 1.84)	0.21	-38.18
		ORIEN	-5.22 (-14.98, 4.84)	0.29	4.44 (-0.09, 10.68)	0.06	-9.66 (-20.38, 0.93)	0.07	-54.18
		PDI	-0.29 (-3.77, 3.21)	0.89	0.88 (-0.81, 2.86)	0.31	-1.17 (-5.11, 2.73)	0.55	-8.93
	EXT1	EMOCI	-6.07 (-13.88, 1.82)	0.12	-0.71 (-3.32, 1.03)	0.43	-5.36 (-13.2, 2.53)	0.17	7.30
		MDI	-1.21 (-5.58, 3.16)	0.59	-0.29 (-1.62, 0.69)	0.56	-0.93 (-5.31, 3.48)	0.71	4.07
		ORIEN	-5.25 (-15.03, 4.47)	0.29	-0.36 (-3.17, 1.91)	0.74	-4.9 (-14.64, 4.9)	0.33	2.51
		PDI	-0.29 (-3.82, 3.2)	0.89	-0.12 (-1.12, 0.7)	0.76	-0.18 (-3.68, 3.34)	0.95	0.59
	KDM6B	EMOCI	-6.07 (-13.91, 1.82)	0.12	1.4 (-1.66, 5.25)	0.37	-7.47 (-15.93, 0.98)	0.08	-17.38
		MDI	-1.22 (-5.59, 3.12)	0.59	0.07 (-1.85, 1.96)	0.94	-1.29 (-6.02, 3.45)	0.59	0.13
		ORIEN	-5.25 (-15.01, 4.52)	0.29	0.84 (-3.16, 5.26)	0.67	-6.09 (-16.57, 4.4)	0.25	-8.21
		PDI	-0.3 (-3.79, 3.16)	0.89	-0.29 (-1.88, 1.11)	0.67	-0.01 (-3.78, 3.76)	0.97	0.66
	GCNT1	EMOCI	-6.13 (-14.13, 1.73)	0.13	-3.52 (-8.09, -0.36)	0.02	-2.61 (-10.56, 5.4)	0.53	50.98
		MDI	-1.23 (-5.59, 3.17)	0.59	-0.63 (-2.62, 0.93)	0.45	-0.59 (-5.26, 4.11)	0.81	12.22
		ORIEN	-5.28 (-15.02, 4.37)	0.29	-2.01 (-6.67, 1.39)	0.28	-3.28 (-13.56, 7.08)	0.54	24.48
		PDI	-0.3 (-3.82, 3.19)	0.89	0.18 (-1.2, 1.64)	0.77	-0.48 (-4.22, 3.29)	0.81	0.00
	RPS29	EMOCI	-6.07 (-13.87, 1.8)	0.12	-0.76 (-3.5, 1.16)	0.45	-5.31 (-13.29, 2.69)	0.18	8.15
		MDI	-1.21 (-5.63, 3.19)	0.59	-0.24 (-1.65, 0.93)	0.68	-0.98 (-5.43, 3.5)	0.69	3.34
		ORIEN	-5.23 (-14.98, 4.58)	0.29	-1.32 (-5.02, 1.01)	0.31	-3.91 (-13.68, 5.89)	0.44	13.88
		PDI	-0.3 (-3.79, 3.14)	0.89	0 (-1, 1.02)	0.98	-0.3 (-3.86, 3.27)	0.89	0.14
	CHTF18	EMOCI	-6.1 (-13.96, 1.79)	0.12	1.34 (-1.5, 5.06)	0.39	-7.44 (-15.69, 0.91)	0.08	-16.22
		MDI	-1.22 (-5.61, 3.15)	0.59	0.49 (-1.14, 2.46)	0.56	-1.71 (-6.32, 2.95)	0.48	-6.66
		ORIEN	-5.26 (-14.95, 4.35)	0.29	0.36 (-3.54, 4.56)	0.84	-5.63 (-15.88, 4.75)	0.29	-3.74
		PDI	-0.29 (-3.76, 3.19)	0.89	-0.48 (-2.07, 0.84)	0.45	0.19 (-3.47, 3.9)	0.90	3.23
	PRDM16	EMOCI	-6.08 (-13.97, 1.84)	0.12	-0.9 (-4.73, 2.46)	0.58	-5.18 (-13.57, 3.29)	0.23	11.93
		MDI	-1.21 (-5.55, 3.17)	0.59	-0.82 (-3.01, 0.99)	0.36	-0.39 (-5.03, 4.3)	0.89	15.82
		ORIEN	-5.25 (-15.04, 4.49)	0.29	-0.91 (-5.56, 3.24)	0.65	-4.34 (-14.69, 6.12)	0.42	9.74
		PDI	-0.29 (-3.78, 3.21)	0.89	-0.79 (-2.6, 0.64)	0.28	0.5 (-3.19, 4.22)	0.79	8.15
	TAPBP	EMOCI	-6.11 (-13.98, 1.71)	0.12	1.63 (-1.33, 5.56)	0.30	-7.74 (-16, 0.66)	0.07	-20.48
		MDI	-1.23 (-5.6, 3.16)	0.60	0.64 (-1.05, 2.71)	0.47	-1.86 (-6.48, 2.83)	0.44	-9.71
		ORIEN	-5.28 (-14.98, 4.38)	0.29	1.95 (-1.72, 6.77)	0.32	-7.23 (-17.42, 3.13)	0.16	-20.35
		PDI	-0.3 (-3.84, 3.16)	0.89	-0.16 (-1.73, 1.35)	0.81	-0.13 (-3.83, 3.62)	0.97	-0.67
	TRAPPC12	EMOCI	-6.11 (-13.97, 1.79)	0.13	-0.73 (-3.63, 1.41)	0.48	-5.37 (-13.48, 2.54)	0.18	8.05
		MDI	-1.21 (-5.6, 3.15)	0.60	0.46 (-0.71, 2.11)	0.44	-1.68 (-6.18, 2.72)	0.47	-5.82
		ORIEN	-5.26 (-15.03, 4.54)	0.29	0.39 (-2.44, 3.69)	0.76	-5.65 (-15.69, 4.15)	0.27	-2.86
		PDI	-0.3 (-3.78, 3.19)	0.89	0.25 (-0.73, 1.5)	0.60	-0.55 (-4.15, 2.96)	0.79	-0.35
T3	*RAB5A*	EMOCI	-1.39 (-9.94, 7.02)	0.77	-1.63 (-5.18, 0.68)	0.17	0.24 (-7.81, 8.67)	0.94	16.10
		MDI	1.61 (-2.89, 6.07)	0.49	-1.13 (-3.27, 0.41)	0.14	2.74 (-1.39, 7.07)	0.22	-22.37
		ORIEN	-0.71 (-11.1, 9.48)	0.91	-1.56 (-5.52, 0.82)	0.23	0.85 (-9, 11.16)	0.85	5.81
		PDI	0.11 (-3.55, 3.73)	0.95	-0.73 (-2.29, 0.29)	0.17	0.84 (-2.62, 4.46)	0.65	1.04
	ICAM5	EMOCI	-1.42 (-9.74, 7.05)	0.76	1.1 (-2.86, 5.45)	0.57	-2.52 (-11.83, 6.79)	0.59	-7.15
		MDI	1.59 (-2.79, 6.09)	0.50	0.4 (-1.71, 2.64)	0.69	1.19 (-3.73, 6.11)	0.63	8.34
		ORIEN	-0.74 (-10.79, 9.6)	0.90	0.68 (-4.15, 5.75)	0.77	-1.42 (-12.67, 9.84)	0.81	0.13
		PDI	0.09 (-3.47, 3.76)	0.93	0.39 (-1.34, 2.24)	0.65	-0.29 (-4.31, 3.73)	0.91	4.22
	GORASP2	EMOCI	-1.45 (-9.77, 6.94)	0.76	0.28 (-1.37, 2.28)	0.73	-1.73 (-9.84, 6.48)	0.70	0.15

	MDI	1.58 (-2.82, 6.05)	0.51	0.09 (-0.69, 1)	0.83	1.49 (-2.82, 5.86)	0.53	0.97
	ORIEN	-0.77 (-10.79, 9.3)	0.90	0.35 (-1.67, 2.79)	0.73	-1.12 (-10.9, 8.77)	0.83	0.78
	PDI	0.08 (-3.52, 3.66)	0.95	0.23 (-0.87, 1.43)	0.66	-0.15 (-3.56, 3.29)	0.96	5.43
TRAPPC6A	EMOCI	-1.42 (-9.72, 7.05)	0.76	1.34 (-3.62, 6.65)	0.59	-2.76 (-12.7, 7.09)	0.58	-8.58
	MDI	1.6 (-2.94, 6.02)	0.50	2.08 (-0.36, 5.06)	0.12	-0.48 (-5.62, 4.61)	0.87	57.95
	ORIEN	-0.72 (-10.87, 9.38)	0.92	3 (-2.76, 9.58)	0.31	-3.73 (-15.64, 8.07)	0.53	-9.51
	PDI	0.08 (-3.52, 3.73)	0.94	-0.73 (-3.06, 1.37)	0.51	0.82 (-3.47, 5.06)	0.70	-5.46
VPS11	EMOCI	-1.46 (-9.73, 6.88)	0.75	2.43 (-0.16, 6.38)	0.08	-3.89 (-12.22, 4.6)	0.37	-18.98
	MDI	1.57 (-2.79, 5.96)	0.51	1.04 (-0.27, 3.04)	0.13	0.53 (-3.92, 5.07)	0.81	25.43
	ORIEN	-0.78 (-10.73, 9.26)	0.90	2.26 (-0.68, 6.8)	0.16	-3.03 (-13.22, 7.36)	0.58	-5.38
	PDI	0.09 (-3.51, 3.72)	0.94	0.37 (-0.75, 1.83)	0.52	-0.29 (-3.99, 3.49)	0.90	2.06
MTA1	EMOCI	-1.43 (-9.65, 7.06)	0.76	0.07 (-1.89, 2.18)	0.92	-1.51 (-9.66, 7.04)	0.74	0.17
	MDI	1.58 (-2.84, 5.99)	0.51	0.27 (-0.61, 1.58)	0.55	1.31 (-2.97, 5.8)	0.58	3.61
	ORIEN	-0.77 (-10.87, 9.3)	0.91	0.74 (-1.22, 3.85)	0.49	-1.51 (-11.27, 8.72)	0.79	0.10
	PDI	0.07 (-3.5, 3.64)	0.95	0.56 (-0.4, 1.94)	0.26	-0.49 (-3.88, 3.07)	0.81	5.60
CCSER1	EMOCI	-1.41 (-9.76, 6.99)	0.76	1.2 (-2.04, 5.03)	0.45	-2.62 (-11.61, 6.51)	0.57	-7.53
	MDI	1.59 (-2.85, 6.03)	0.50	0.73 (-0.97, 2.78)	0.38	0.86 (-3.88, 5.67)	0.71	16.53
	ORIEN	-0.73 (-10.91, 9.36)	0.92	1.74 (-2.15, 6.48)	0.36	-2.47 (-13.29, 8.51)	0.65	-3.49
	PDI	0.1 (-3.51, 3.72)	0.93	0.51 (-0.89, 2.16)	0.45	-0.41 (-4.29, 3.52)	0.85	3.39
EXT1	EMOCI	-1.4 (-9.97, 7.1)	0.75	0.68 (-1.16, 3.35)	0.50	-2.08 (-10.33, 6.37)	0.64	-0.16
	MDI	1.59 (-2.85, 6.01)	0.50	0.24 (-0.61, 1.44)	0.60	1.36 (-3.05, 5.87)	0.56	3.92
	ORIEN	-0.73 (-10.81, 9.43)	0.92	0.41 (-1.41, 2.97)	0.67	-1.14 (-11.23, 9.2)	0.84	0.22
	PDI	0.1 (-3.51, 3.72)	0.93	0.15 (-0.51, 1.08)	0.66	-0.06 (-3.67, 3.64)	1.00	0.93
KDM6B	EMOCI	-1.43 (-9.77, 7.1)	0.76	-0.11 (-3.83, 3.46)	0.94	-1.32 (-10.51, 7.81)	0.78	1.29
	MDI	1.58 (-2.83, 6.04)	0.51	-0.54 (-2.62, 1.23)	0.55	2.12 (-2.72, 6.93)	0.37	-10.70
	ORIEN	-0.75 (-10.78, 9.53)	0.91	-0.85 (-5.47, 3.26)	0.67	0.09 (-10.98, 11.08)	0.95	0.61
	PDI	0.09 (-3.53, 3.75)	0.93	-0.41 (-2.09, 1.05)	0.59	0.49 (-3.46, 4.42)	0.79	-2.63
GCNT1	EMOCI	-1.47 (-9.76, 6.87)	0.74	-1.88 (-5.89, 1.1)	0.23	0.41 (-7.42, 8.47)	0.89	26.02
	MDI	1.58 (-2.82, 6)	0.51	-0.41 (-1.83, 0.44)	0.43	1.98 (-2.42, 6.51)	0.40	-4.43
	ORIEN	-0.77 (-10.72, 9.29)	0.90	-1.18 (-4.64, 1.01)	0.33	0.4 (-9.58, 10.67)	0.91	4.15
	PDI	0.09 (-3.51, 3.78)	0.93	0.08 (-0.72, 1)	0.84	0.01 (-3.62, 3.75)	0.97	0.77
RPS29	EMOCI	-1.4 (-9.99, 7.1)	0.75	-0.93 (-3.88, 0.86)	0.35	-0.47 (-8.62, 7.96)	0.93	7.12
	MDI	1.59 (-2.87, 6.01)	0.50	-0.32 (-1.7, 0.57)	0.50	1.92 (-2.43, 6.41)	0.41	-3.36
	ORIEN	-0.71 (-11.09, 9.47)	0.91	-1.34 (-5.12, 1.02)	0.29	0.63 (-9.13, 10.73)	0.88	4.91
	PDI	0.09 (-3.47, 3.78)	0.93	-0.07 (-1, 0.74)	0.87	0.16 (-3.41, 3.86)	0.91	-0.08
CHTF18	EMOCI	-1.43 (-9.72, 7.06)	0.76	0.04 (-2.32, 2.46)	0.96	-1.47 (-9.89, 7.28)	0.75	0.14
	MDI	1.59 (-2.79, 6.09)	0.51	-0.03 (-1.29, 1.22)	0.96	1.61 (-2.83, 6.23)	0.50	-0.14
	ORIEN	-0.74 (-10.75, 9.54)	0.90	-0.48 (-3.65, 2.23)	0.71	-0.26 (-10.4, 10.28)	0.99	0.83
	PDI	0.1 (-3.54, 3.7)	0.94	-0.45 (-1.79, 0.42)	0.34	0.55 (-3.04, 4.28)	0.76	-0.54
PRDM16	EMOCI	-1.4 (-9.99, 7.06)	0.75	-1.03 (-4.07, 0.76)	0.32	-0.38 (-8.56, 8.14)	0.95	7.66
	MDI	1.6 (-2.94, 6.08)	0.50	-0.55 (-2.16, 0.4)	0.32	2.15 (-2.18, 6.64)	0.36	-8.15
	ORIEN	-0.72 (-10.9, 9.33)	0.92	-1.05 (-4.52, 1.12)	0.39	0.33 (-9.59, 10.66)	0.93	2.51
	PDI	0.1 (-3.54, 3.69)	0.94	-0.37 (-1.61, 0.4)	0.39	0.47 (-3.08, 4.17)	0.79	0.08
TAPBP	EMOCI	-1.43 (-9.75, 7.06)	0.76	0.17 (-2.34, 2.9)	0.88	-1.6 (-10.1, 7.16)	0.73	-0.54
	MDI	1.58 (-2.78, 6.07)	0.51	0.01 (-1.35, 1.41)	0.97	1.57 (-2.92, 6.2)	0.51	0.37

	ORIEN	-0.75 (-10.8, 9.52)	0.90	0.42 (-2.53, 3.8)	0.77	-1.17 (-11.42, 9.39)	0.85	0.12
	PDI	0.1 (-3.5, 3.74)	0.93	-0.3 (-1.61, 0.72)	0.54	0.4 (-3.25, 4.17)	0.82	-1.09
TRAPPC12	EMOCI	-1.45 (-9.82, 6.87)	0.75	-0.36 (-2.53, 1.1)	0.66	-1.09 (-9.6, 7.45)	0.81	1.43
	MDI	1.59 (-2.8, 6.07)	0.50	0.16 (-0.65, 1.26)	0.71	1.43 (-3.07, 5.94)	0.53	2.31
	ORIEN	-0.75 (-10.71, 9.47)	0.90	0.18 (-1.66, 2.45)	0.83	-0.93 (-11.24, 9.41)	0.87	0.38
	PDI	0.09 (-3.48, 3.75)	0.94	0.05 (-0.62, 0.84)	0.87	0.04 (-3.65, 3.74)	0.96	0.73

Chapter 5 Discussion

Dissertation Objectives

Lead (Pb) exposure is a persistent problem in the United States and around the world. Pregnant women and their developing fetuses are particularly vulnerable to Pb because both past and present exposures can affect gestational growth and development. The health impacts of prenatal Pb exposure can be framed with the Developmental Origins of Health and Disease (DOHaD) hypothesis [1]. Accumulating evidence for DOHaD includes potential mechanisms such as epigenetic reprogramming via DNA methylation (5mC) and, less studied, hydroxymethylation (5hmC), linking developmental exposures to adverse childhood and adulthood outcomes. In an effort to address these questions, in this dissertation I aimed to determine if perinatal Pb exposure alters DNA 5mC and 5hmC levels, assessed through both whole-genome and candidate gene methods, and determine if DNA methylation mediates the relationship between prenatal Pb exposure and adverse neurodevelopment (**Figure 1.2)**.

For the entire dissertation, I utilized a longitudinal human birth cohort with known perinatal exposures to Pb, biological samples at birth and into adolescence, and measures of infant neurodevelopment: Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) [2]. In Aim 1, I tested whether early-life exposure to Pb alters the epigenome at birth in umbilical cord blood (UCB), as has been suggested in previous human, animal model, and *in vitro* studies [3-7], but expanded to include the independent effects of trimester-specific exposures and cumulative exposure over the gestational

period. In the Aim 2, I evaluated gene-specific DNA 5mC and 5hmC in adolescent whole blood from the same cohort to determine whether the association of gestational Pb exposure and epigenetics persists into adolescence. I expanded from previous literature to determine if 5hmC is associated with the exposure. In Aim 3, applying the UCB DNA methylation from the first aim, I analyzed whether DNA methylation at birth would mediate the association between trimester-specific Pb exposure and neurodevelopmental outcomes, in line with the DOHaD paradigm.

Summary of research findings

The findings of this research (**Figure 5.1**) provide insights into epigenetic responses following developmental Pb exposure, indicating surprising complexities that should be taken into account in interpreting human and animal studies. In Aim 1 (Chapter 2), I found that prenatal Pb exposure was associated with altered epigenetic patterns at birth and the alterations were trimester-specific. Epigenome-wide analyses identified three differentially methylation positions (DMP (i.e., CpG site)) that mapped to *RAB5A, EXT1,* and a non-genic region and one differentially methylated region (DMR) within *PDGFRL* associated with first trimester (T1) BLLs, one DMP and one DMR with third trimester (T3) BLLs that were both within an intergenic region, and two DMPs and one DMR with tibia bone Pb, a measure of cumulative exposure across the entire gestational period, which mapped to *LRFN1*, a non-genic site, and *TRHR*, respectively. The number of associated DMPs were quite small, indicating that at least with the sample size present and the range of BLLs, there wasn't any observed extensive DNA methylation response. Further studies are needed to determine if Pb differentially modifies the expression of genes regulated by these DMPs and DMRs as RNA was not

available in ELEMENT infant cord blood. Additionally, performing an additional experiment encompassing a larger sample size and large variability in Pb exposure could enable detection of more extensive changes. Pathway analysis identified multiple gene pathways enriched for differential methylation with several pathways overlapping between T1 and T2 including nodal signaling, neurological system processes, and neurotransmitter activity-related genes. This suggests that trimester-specific prenatal Pb exposure may modify DNA methylation profiles at birth, and this should be considered as one potential mechanism underlying Pb's neurotoxic effects during development.

In Aim 2 (Chapter 3), I identified associations of sex-specific and trimester-specific prenatal Pb exposure with DNA 5mC and 5hmC levels, independently, at multiple cytosine-phosphate-guanine (CpG) sites within gene regions previously associated with prenatal Pb exposure in human whole blood within the same cohort as Aim 1 but at the adolescent-age timepoint (11-17 years old). I build upon Aim 1 by investigating genes with identified alterations associated with T1 BLLs within that aim, *RAB5A, NINJ2, HCN2,* and *TPPP*, but included a later timepoint and distinguished between 5mC and 5hmC. I provided evidence for variable 5hmC in human whole blood with 12.5% of the study participants displaying 5hmC levels measuring above 15% in at least one CpG site measured. I also show that prenatal Pb exposure's alterations of gene-specific 5mC and 5hmC levels in blood are stable from birth into adolescence, which is discussed in more detail in the next section. To strengthen these findings, I quantified gene expression in a subset of individuals and showed that increased 5mC was negatively correlated with *NINJ2* expression, whereas increased 5hmC was positively correlated, which follows previous research [8, 9]. Overall, this provides

evidence to consider 5hmC as a regulatory response mechanism to environmental exposure and should be explored further. Finally, I detected significant sex-specific associations. I detected more statistically significant Pb-induced 5mC and 5hmC changes among females as compared to males.

In Aim 3 (Chapter 4), I elucidated a potential mechanism linking developmental Pb exposure to early-life adverse neurodevelopmental outcomes. I performed a pairwise mediation analysis to identify DNA methylation's mediating effects on the association between exposure and outcome. I provided evidence that epigenetics may contribute as a potential biological mechanism underlying Pb's adverse health effects on neurodevelopment, specifically at 24-months of age in both a mediating and suppressive manner. Little evidence was shown for significant reductions in neurodevelopmental scores by Pb exposure at the 12-month timepoint. Our pilot study builds upon previous research by considering DNA methylation at functionally-relevant genes as a potential mechanism underlying Pb's adverse effects on neurodevelopment.

This dissertation research corroborates previous human, animal, and *in vivo* literature demonstrating associations between prenatal Pb exposure and epigenetics [3-7], and builds on it by incorporating multiple biomarkers of exposure, utilizing a longitudinal study design, two epigenetic marks (5mC and 5hmC), as well as investigating the mediating effect of the epigenome on neurodevelopmental outcomes at two infancy timepoints. Additional studies, either animal or *in vitro*, are needed to characterize the underlying biology to explain these observations.

Synthesis of evidence for proposed mechanism

In the epigenome-wide analysis performed in Aim 1, statistically significant DMPs identified were mostly hypomethylated with a couple being hypermethylated, which follows the proposed model that Pb inhibits DNMTs from binding and reduces methyl marks (**Figure 1.1**). However, the statistically significant DMRs were hypermethylated, which may indicate that cells are responding to the exposure by silencing gene promotors or enhancers of certain genes. Studies have provided evidence for both increases and decreases in methylation at specific DNA sequences serving as biomarkers for a variety of diseases, including neurodevelopmental [10-16]. Globally, the effect estimates between Pb and DNA methylation were mostly hypermethylated for the blood Pb measures (T1: 67.7% sites positively associated with Pb; T2: 66.3%; T3: 61.4%), but mostly hypomethylated for the patella and tibia Pb measures, 54.7% and 63.7%, respectively. These findings were similar to a study performed in the US within the Early Autism Risk Longitudinal Investigation (EARLI) cohort (n=97), which found global pregnancy DNA hypermethylation (~55.8% of CpG sites tested) and as well as site-specific hypermethylation associated with T1 and T2 BLLs (mean: 0.4 μg/dL, lower than our current study) [17]. The significant site-specific CpG's were related to nervous system development and calcium ion binding, similar to our study which also had statistically significant genes annotate back to pathways involved in various neurological and neurodevelopmental functions. In contrast, a study utilizing newborn dried blood spots from the Michigan-based Healthy Families Project (n=96) identified mostly hypomethylation with increase Pb exposures (mean: 0.78 μg/dL) [18]. Overall, there seems to be evidence for some hypomethylation and hypermethylation with Pb,

depending on the genes and/or gene regions assessed, showing that there isn't clear evidence yet for Pb's impact on DNA methylation (**Table 1.1**). Further studies are needed to strengthen previous findings. In this dissertation, these genes were investigated further in Aims 2 and 3. *RAB5A* plays a role in localizing early endosomes and intercellular membrane trafficking and fusion, and perturbations around the endosomal dynamics may underlie neuronal dysfunction and degeneration in various neurodegenerative diseases [19]. The primary role of *EXT1* is heparan sulfate biosynthesis, which regulates multiple biological processes including cell proliferation, growth factor signaling, and mammalian neuronal development [20-23]. *LRFN1* promotes neurite outgrowth and synapse formation in hippocampal neurons and has a role in the developing nervous system with involvement in regulating and conserving excitatory synapses [24-26]. *TRHR* activates the phosphatidylinositol-calcium-protein kinase C transduction pathway, which signals synthesis, secretion, and bioactivity of the thyroid stimulating hormone in the pituitary gland [27, 28]. *PDGFRL* does not have a known function in development but it does encode a tumor suppressor that inhibits the growth of colorectal cancer cells [29]. These gene-specific findings suggest that epigenetic mechanisms are being modified by prenatal Pb during critical developmental periods and DNA methylation should continue to be explored as a potential mechanism underlying Pb's neurodevelopmental effects. In Aims 2 and 3, I also investigated genes that were not significantly associated with prenatal Pb in Aim 1 but had log fold changes in methylation per unit increase in prenatal Pb at or above 5% or were involved in various neurological functions; Aim 2: *TPPP, NINJ2,* and *HCN2*; Aim 3: *KDM6B*,

GCNT1, *RPS29*, *CHTF18*, *PRDM16*, *TAPBP*, *TRAPPC12*, *ICAM5*, *GORASP2*, *TRAPPC6A*, *VPS11*, *MTA1*, and *CCSER1*. They will be discussed below.

In the targeted gene sequencing analysis within Aim 2, all three trimester BLLs were associated with hypermethylation within *HCN2*, a gene known to regulate dopamine neuronal activity [30]. All three trimester BLLs, as well as patella bone Pb, were associated with hyper-hydroxymethylation within *NINJ2*, a transmembrane protein that mediates cell-to-cell and cell-to-extracellular matrix interactions during development, differentiation, and regeneration of the nervous system by promoting neurite growth [31]. In contrast, T3 BLLs were associated with hypomethylation, but this opposing direction is expected because studies have shown that as 5mC levels decrease, 5hmC would subsequently increase [32]. Of the significant findings among males, prenatal exposure was associated with hypermethylation in *TPPP* in adolescence, which plays a role in the integrity of the microtubule network as a potential hallmark of synucleinopathies (neurodegenerative diseases characterized by abnormal accumulation of TPPP/p25 and alpha-synuclein) [33]. Further, there is evidence that sex can influence the severity of Pb neurotoxicity [6, 34, 35]. Although, based on magnitude, direction and CI overlap of the associations between Pb and 5mC or 5hmC in sex-stratified analyses in Aim 2, we cannot state with certainty that these effects are truly sex-specific. For example, T2 BLLs were positively associated with *NINJ2* 5hmC in females (β=0.48 (95% CI: 0.10, 0.86)), while the association was close to null for males (β=0.07 (95% CI: –0.20, 0.34)), and the associations between 5mC with T1 BLLs and patella Pb were negative for females and remained close to null for males, but confidence intervals overlapped. These data are inconsistent with previous studies which showed Pb-associated DNA

methylation changes identified sex-specific effects, mostly in females [6, 36]. Yet, collectively this dissertation and other studies suggest that sex, as well as exposure timing and exposure dose, may influence an individual's susceptibility to the effects of Pb.

After completing Aim 2, a question arose on whether the associations between Pb and DNA methylation that I identified at birth (Aim 1) were consistent in direction and magnitude in adolescence (Aim 2). Since Aim 1 and Aim 2 were conducted with different methylation quantification platforms, I decided to utilize previously quantified adolescent-age DNA methylation genome-wide profiles from the same platform as Aim 1, MethylationEPIC array, to determine if the changes at birth persisted into adolescence. Of the samples available with data at both time points, we were able to compare 72 matched UCB and adolescent whole blood samples. First, I pulled all the previously normalized and batch corrected beta values (for Methods, see Chapter 2: Methods – Epigenetic Analysis) for each sample from the CpGs identified in Aim 1 and Aim 2 as statistically association with first trimester BLLs or effect estimates greater than |5%| when analyzing first trimester BLLs and are linked to neurodevelopment of this dissertation (cg17138393, cg00984923, cg25353752, cg26371957, cg19692784, cg14911689, cg05578102, cg26654770, cg01201512, cg06657917, cg03390844, cg01328348, cg00002033, cg03463208). Beta values represent proportion methylated. We then plotted the beta values between the two timepoints for the CpG sites listed above that mapped back to genes (**Figure 5.2** and **5.3)**. Not to my surprise, some genes contain consistent levels of methylation between birth and adolescence while others do not [37, 38]. DNA methylation regulates expression and not all genes need to be

expressed at all points in development. For example, *LRFN1* has low methylation at birth, meaning the gene is likely expressed, but high methylation later in life (**Figure 5.2, top left**). This is likely due to *LRFN1*'s role in neurite growth while the nervous system is developing. This function isn't necessarily needed later in life. These predictable changes in DNA methylation are termed 'age-related methylation,' and these changes have important implications that may underlie later-life disease development through transcriptional control and expression [39-41].

Next, I pulled all effect estimates from the linear regression models previously performed (for Methods, see Chapter 2: Methods – Statistical analyses) from the matched UCB and adolescent-aged samples. I ran Pearson's product-moment correlation to quantify the correlation of the relationship between matched effect estimates of the association between prenatal Pb and DNA methylation at birth and in adolescence. Globally (at all >700,000 CpG sites), the effect estimates were slightly correlated (T1: R=0.29; T2: R=0.15; T3: R=0.12; Patella: R=0.13; Tibia: R=0.12). Although, when I looked at the fourteen CpG sites of most interest related to Aim 1 and Aim 2, I showed that prenatal Pb's effect on DNA methylation is highly correlated from birth into adolescence for each biomarker of exposure except tibia (**Figure 5.4**). Interestingly, T1 BLLs effect estimates had the strongest correlation (R=0.96) from birth to adolescence and the association among all CpG's from birth to adolescence was stable, potentially suggesting that perturbations occurring during the first trimester are reprogramming the epigenome and these changes are remaining through adolescence, at least at these 15 CpG sites. Thus, I provide evidence that Pb's toxicoepigenomic effects are stable at some but not all CpG sites.

Finally, the mediation analysis provided evidence that gene-specific DNA methylation at birth within several genes mediated or suppressed the association between T2 measures of prenatal Pb and neurodevelopmental measures at 24-months of age; the strongest evidence was with *CCSER1* methylation suppressing the association and *GCNT1* methylation mediating with both showing percent suppressed/mediated around 50% within the model. Although *CCSER1* is frequently identified in human cancers, it has recently been associated with attention deficit hyperactivity disorder after expression was discovered in the cerebellum [42, 43]. *GCNT1* acts like a cell surface marker indicating whether a T-cell has received Notch signaling, which stimulates proliferation during neurogenesis and plays a key role in regulation in embryonic development [44-46]. Mediating effects were also identified with *VPS11*, which alterations have been hypothesized in contributing to progressive developmental delays by attenuating the degradation of plasma membrane receptors [47]. This pilot study provides preliminary evidence into how epigenetic reprogramming events relate to specific biological pathways influencing fetal neurodevelopment and builds upon previous research on prenatal Pb and its association with decreased childhood IQ and neurobehavioral problems.

Although epigenomic changes in early gestation would be expected to propagate across all tissues, it is still important to consider tissue-specificity when conducting differential methylation studies. To fully synthesize the proposed mechanisms, I decided to utilize a renowned blood-brain epigenetic database to compare our blood results with brain epigenomic profiles [48]. From among the 15 CpG sites I studied extensively in this dissertation, 8 were include in the database. Of these 8 CpG sites, 5 were moderately to

highly correlated between blood and the prefrontal cortex (cg14911689: r=0.88, p=8.81e-25; cg26654770: r=0.88, p=1.93e-24; cg01201512: r=0.86, p=4.7e-23; cg06657917: r=0.72, p=5.84e-13; cg26371957: r=0.44, p=9.96e-5), entorhinal cortex (cg14911689: r=0.84, p=1.39e-19; cg26654770: r=0.85, p=1.23e-20; cg01201512: r=0.82, p=1.74e-18; cg06657917: r=0.79, p=5.29e-16; cg26371957: r=0.48, p=2.22e-5), superior temporal gyrus (cg14911689: r=0.89, p=6.54e-27; cg26654770: r=0.90, p=1.46e-27; cg01201512: 0.87, p=2.0e-24; cg06657917: r=0.80, p=5.93e-18; cg26371957: r=0.38, p=0.66e-3), and cerebellum (cg14911689: r=0.78, p=6.78e-16; cg26654770: r=0.70, p=1.62e-11; cg01201512: r=0.52, p=4.21e-6; cg26371957 (r=0.15, p=0.21) and cg06657917 (r=0.038, p=0.75) were not correlated with the cerebellum) brain regions. DNA methylation in umbilical cord blood of 3 CpG-sites were not correlated with any brain regions (cg25353752, cg00002033, cg03463208). This database is a great first resource to see which sites correlate between brain and blood, but this tool does not account for exposures and the samples are from individuals over 50 years of age. Since 5 of the 8 CpG sites were correlated, this suggests that some biomarkers in blood are possible for certain genes. Brain samples should be the primary tissue of interest to better understand the underlying pathological processes behind prenatal Pb exposure and neurodevelopmental molecular processes.

Significance of findings

Despite multiple public health initiatives aimed at reducing Pb exposure, such as the removal of Pb from gasoline, paint and other products, it remains a major concern in the United States (US) and around the world. In fact, it has been estimated that one-third of all US homes have Pb-based paint with a high percentage of these homes being

rental properties [49]. Further, there is growing fear about exposure risk due to the aging infrastructure, especially after a highly publicized contamination from failed Pb pipes in Flint, MI, which spiked testing that revealed other US cities with similar problems [50, 51]. In middle- and low-income countries, additional contamination sources include Pb-glazed ceramics and Pb-acid battery production and recycling [52]. These are just some sources of unwanted environmental Pb exposure, which are especially dangerous for pregnant women, children, and other susceptible populations. While research continues to suggest that there is no safe level of Pb, studies are still conducted to understand the impacts of low-level Pb exposure, which is considered to be <5 μg/dL in blood, mainly focusing on early stages of development. It has been known for centuries that Pb has adverse neurological effects, but current research does not fully answer the question around the epigenetic mechanisms that underlie Pb-induced damaging impacts on cognitive development. There is a growing body of evidence that environmental exposures play a role in cognition. As such, biomarkers of the developmental environment, including the epigenome, have potential to provide valuable information about cognitive delays.

The evidence for DOHaD, which states that environmental insults ranging from nutrient starvation to active exposure to toxicants cause damages to the developing body which can manifest once the organism is older, is quite strong for some exposures [1]. Thus, it is plausible that DNA methylation or other epigenetic changes is a mechanism by which early life Pb exposure leaves a stable mark that modifies biological function manifesting in adverse outcomes, including neurological outcomes, later in life. In an effort to better understand the mechanisms of action for developmental

Pb exposure, recent studies have investigated the epigenetic effects, showing that developmental Pb can alter genome-wide, imprinted genes, and gene promotor-specific methylation in human models [4, 6, 53]. However, these studies failed to include biomarkers of both trimester-specific and cumulative exposure (i.e., maternal bone Pb). Bone Pb is an excellent proxy for long-term cumulative exposure over the gestational period [54, 55]. Although I did not identify similar associations between the two bone Pb measures with DNA methylation and hydroxymethylation, this is anticipated. Patella stored Pb has a half-life ranging from months to years [55], whereas Pb in tibia has a residence time of 25-30 years [54]. While both serve as proxies for gestational Pb exposure, the relationship between maternal bone Pb and fetal exposure dose throughout pregnancy is influenced by the differences in half-life and bioavailability (i.e., following bone turnover). Here, I followed-up on previous studies, providing preliminary evidence that gene-specific DNA methylation changes occur, with specific effects taking place during particular timepoints. Further, DNA methylation changes at birth persisted at some but not all genes into adolescence. Given the precise biological processes that occur during fetal development, modified regulations of neurological-related genes have the potential to adversely impact cognitive health. As such, our results show Pb-related differential methylation may represent an epigenetic mechanism by which Pb impacts cognition.

While previous human and animal exposure studies have provided insight into epigenome-environment interactions, they have primarily used the standard bisulfite sequencing methods, which collectively measures both 5mC and 5hmC. These two epigenetic marks typically have opposing associations with gene transcription and expression [56]. Failing to distinguish between the two independent marks might result in

inaccurate conclusions. Previous work has demonstrated that 5hmC is tissue-specific with highest levels present in the brain and low levels in whole blood [57]. Here, I extended our study to include 5mC, 5hmC, and total methylation (5mC+5hmC) to better estimate which exact epigenetic mark(s) Pb may modify. Not only was I able to detect 5hmC in human whole blood, but I also provided evidence for Pb-induced gene-specific and sex-specific changes with each epigenetic mark. However, I do not provide evidence for biological mechanisms that might explain the epigenetic changes that are occurring. Additional studies are needed to expand beyond our findings to include more diverse study populations, different tissue types for epigenetic analysis, use of animal studies to control confounders, and larger sample sizes with adequate statistical power in order to make accurate conclusions around the conclusions present.

Impact and innovation

This is the first study to investigate the mediating effects of DNA methylation on the association between prenatal Pb exposure and neurodevelopment outcomes in infancy. Historically, studies have either looked at Pb's effects on DNA methylation or Pb's effects on neurodevelopmental outcomes. Here, I combined the two in order to paint a better picture of the biological mechanisms. Our findings suggest that the impact of developmental exposures to Pb on neurodevelopmental measures, especially emotional scores, may be both mediated and suppressed by DNA methylation, depending on the gene. This may provide evidence for dynamic biological processes, meaning that epigenetic regulation of many genes could be impacted by Pb's toxic effects, but depending on the function of the gene and the direction and magnitude of the effect, these changes could contribute to Pb toxicity, protect against some of Pb's

effects, or have no meaningful biological effect. Therefore, this work suggests that more research investigating the health impacts of developmental Pb is needed to better understand the role of epigenomics in Pb's long-term effects.

The analysis of multiple biomarkers of prenatal Pb exposure allowed us to narrow-down exposure timing from the broad 'prenatal' period to 'trimester-specific,' for which future research could illuminate on prevention and intervention methods in a more time-specific manner. I expected to see more significant results associated with first trimester exposures because neurogenesis occurs at an astonishing rate followed by neuronal proliferation and migration during that time [58, 59]. Although other important processes, such as dendrite formation, synapses formation and circuit organization, neuronal differentiation, axonal elongation, and myelination, occurring during the later gestational period [60-65]. The longitudinal aspect of our study allowed us to characterize the epigenome's ability to maintain stability from birth into adolescence and further estimate more accurate exposure-outcome relationships by developmental timepoint. The quantification of two epigenetic marks, 5mC and 5hmC, allowed us to identify whether environmental exposures alter 5mC, 5hmC, or both. Most current methods collectively measure both without distinguishing between the two; but recent studies suggest that the functional role of 5hmC is distinct from 5mC [66]. I provide evidence for this widely-debated mark, 5hmC, having its own distinct role into the mechanisms behind Pb-induced epigenetic alternations. Additional studies are needed to differentiate the two marks and determine if previous findings combining the two epigenetic marks are making accurate conclusions based on their combined method (5mC+5hmC) outcomes.

This study is also the first to examine the independent associations of 5mC and 5hmC with prenatal Pb exposure. Previous work has shown that gene transcription and expression differs between these two epigenetic marks [56]. For example, increases in 5mC results in decreases in expression, whereas increases in 5hmC results in increases in expression. Our results showed this opposing trend within *NINJ2*. When assessing prenatal Pb exposure, as 5mC increased, 5hmC subsequently decreased. Further, this is one of a few studies to isolate 5hmC in human whole blood and the only study to compare levels of 5hmC to prenatal Pb exposure in humans. This work helps to interpret results reported in past epidemiological studies and informs the planning of future studies because it shows that the standard bisulfite sequencing methods (combines 5mC+5hmC) may not provide accurate conclusions regarding the distinct epigenetic marks. It is also informative to future studies, providing insight into more rigorous data collection when measuring DNA methylation and offering a novel method for quantitative 5hmC measures in human whole blood.

Limitations

Despite the innovative and novel aspects of this study, there are also aspects of this dissertation that limit the ability to conclusively prove underlying biological mechanisms. The results of this study were associative in nature as opposed to causal but helped us identify genes of interest for further exploration. In terms of other limitations, I was unable to utilize positive hydroxymethylation controls for Aim 2 pyrosequencing. There are currently no commercially available hydroxymethylation controls for the oxBS pyrosequencing method. Although, I did run eight oxBS samples in triplicate within two gene regions to measure the coefficient of variation (<10%) for

the 5mC measures and utilized human methylated (0%) and unmethylated (100%) controls (I.e., 5mC+5hmC) in order to account for this limitation. Given how recently 5hmC has emerged as a topic of research interest, precise differences in how gestational Pb exposure alters 5hmC epigenome-wide in both mice and humans remains unclear. Our research team has previously thought of this and is currently studying this in mice.

Additionally, the low number of human samples in all aims limited statistical power. Specifically, the small sample sizes within Aim 1 and Aim 3 prevented us from performing sex-specific analyses. The small number of families that had UCB collected originally and loss to follow-up played a major role in reducing the sample size. Also, the environment of the recruited human cohort was not controlled which is an inherent limitation in any epidemiology study, meaning that background exposure to various environmental factors may be confounding the reported results. Further, the tissue collected, blood, is a limitation given our primary tissue of interest, brain. Although, given our first trimester results, blood could potentially be a surrogate tissue for target tissues following early-life epigenetic perturbations. However, more research is needed to confirm this. Human brain samples are inherently rare and can only be obtained post-mortem making longitudinal studies impossible. Therefore, I recommend future studies to incorporate larger sample sizes in order to increase statistical power and account for environmental variability and population heterogeneity, as well as animal models with controlled environments, so we may more robustly identify key genes and molecular pathways linking Pb exposure to outcomes.

Recommendations for future research

This research attempte to integrate data from multiple timepoints within a longitudinal birth cohort to investigate the contribution of toxicant-mediated DNA methylation perturbations to adverse neurodevelopmental outcomes in early-life. Even though I found a number of significant results, interpretation of these data is limited by the aforementioned limitations, but these data are suggestive of the role of DNA methylation and hydroxymethylation in Pb toxicity and may help inform the design of additional studies. Replication of these findings in diverse human cohorts is needed to fully understand the risks of development Pb exposures.

The targeted epigenomic analysis in Aim 2 provided valuable information regarding the distinct roles of 5mC and 5hmC, even though it only covered four gene-regions out of the entire genome. Bisulfite conversion, used in Aim 1 and 3 which is the most widely used method to measure DNA methylation, does not distinguish between 5mC and 5hmC. In future studies, DNA hydroxymethylation analyses, using technologies such as antibody pull-down methods (hydroxymethylation DNA immunoprecipitation sequencing (hMeDIP-seq)), oxidative bisulfite sample conversion (paired with MethylationEPIC beadarray) or other methods to distinguish the two epigenomic marks, would provide insight into global, epigenome-wide hydroxymethylation, and targeted changes. Given the novelty of the findings in Aim 2, this epigenetic mark needs to be quantified in future environmental epigenetic studies in order to provide the most accurate insights.

Since it is difficult to control for all potential confounding effects within human longitudinal cohorts, animal model studies of Pb exposure are needed. Murine studies

have careful control measures, where exposures represent the only difference between two groups of mice (exposed versus unexposed). In addition, murine studies allow research to define the exposure period, meaning when the exposure starts and stops. This way researchers can ensure that gestational exposure is occurring during fetal development and no other exposures are confounding the effects. Moreover, it is still important to consider tissue-specificity when conducting differential methylation studies. Murine studies allow access to tissues that are not necessarily available in humans, like brain which is the primary tissue of interest in terms of Pb toxicity. Additional cross-species and cross-tissue studies are needed.

In fact, in collaboration with the Toxicant Exposures and Responses by Genomic and Epigenomic Regulators of Transcription (TaRGET II) consortium [67], we are assessing how epigenomic marks correlated between surrogate tissues and target tissues using murine models in order to address this cross-tissue and interindividual variation in epigenomic profiles in disease susceptibility [67]. Dams, which are genetically invariant mice 93% identical to C57BL/6J strain, were assigned to control or Pb-acetate (32ppm) water two weeks prior to mating. Exposure continued throughout gestation and lactation until weaning at post-natal day 21 (PND21), upon which all offspring consumed control water. *In utero* exposure, thus, occurred in offspring throughout fetal development. We collected whole blood and brain of 5-month-old male and female mice and we will perform whole-genome bisulfite sequencing (WGBS) and hydroxymethylation DNA immunoprecipitation sequencing (hMeDIP-seq) in hopes to provide valuable insight into the effects of developmental exposure on epigenomic reprogramming in a tissue-, sex-, and cell-type-specific manner.

Overall conclusions

In this dissertation project, I utilized a longitudinal human birth cohort, ELEMENT, of perinatal exposures to Pb to provide evidence supporting the hypothesis that developmental exposures to Pb have negative impacts on cognition in early life, and to investigate a novel molecular mechanism underlying this relationship - epigenetics. I aimed to fill this critical gap in the literature by assessing prenatal Pb exposure effects on DNA 5mC and 5hmC patterns, and its association with neurodevelopmental outcomes. This is the first longitudinal human study to investigate the mediating role of epigenetics at birth on the relationship between developmental Pb and early-life neurodevelopmental measures, and I revealed both mediating and partial reversal (I.e., suppressive) effects. I further reported trimester-specific associations between Pb exposure and DNA methylation, as well as sex-specific effects. Incorporating the independent measures of 5mC and 5hmC was another unique aspect of this research. I found that prenatal Pb exposure altered the 5mC and 5hmC, individually, and emphasizes the importance of distinguishing the two in toxicoepigenetic studies. The contrast in these two epigenomic marks I observed might be one explanation for the discrepancies in previous research regarding DNA methylation's relationship with gene expression, which is normally inverse.

In terms of public health, I show that there is still more research needed to determine the various exposure doses and timing that may impact DNA methylation. Nonetheless, this dissertation may provide evidence for specific genes or gene pathways to investigate that might explain prenatal Pb-induced later life health outcomes. DNA methylation differences that I show should be followed up within this

cohort to determine if these changes continue to persist into adulthood; and if they changes persist, determine whether they are associated with adverse neuro-related outcomes, such as Alzheimer's. This work contributes to the growing body of evidence calling for the prevention of all Pb exposure. But if intervention is the case, we also provide potential genes worth pursuing. These results suggest that environmental factors play a key role in the developmental origins of health and disease and that DNA methylation may play a mediating and/or suppressive role when assessing exposure-outcome models. This idea has important implications for public health policies related to gestational exposure prevention and intervention through lowering blood Pb levels even further, specifically to no exposure.

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Figures

Figure 5.1: Conceptual model summarizing the dissertation aims and results. Each aim is indicated by colored dashed lines (Aim 1 = blue, Aim 2 = purple, Aim 3 = green). Biomarkers and variables considered within statistical modeling are indicated within each grey box and results for each aim are stated within blue boxes.

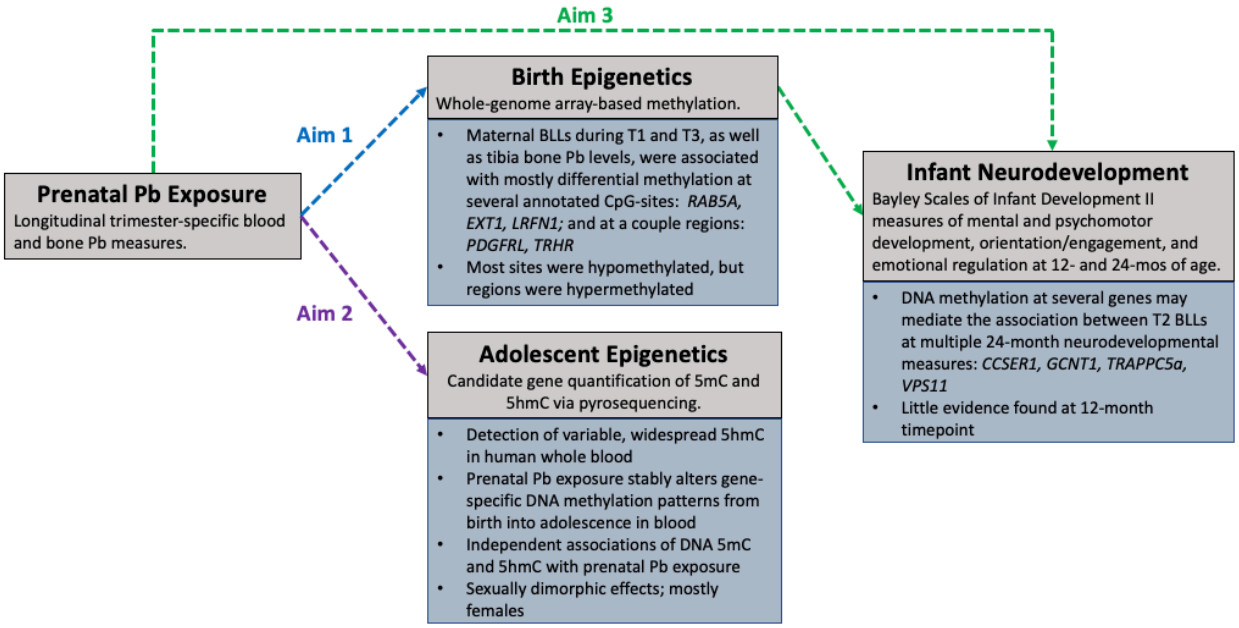
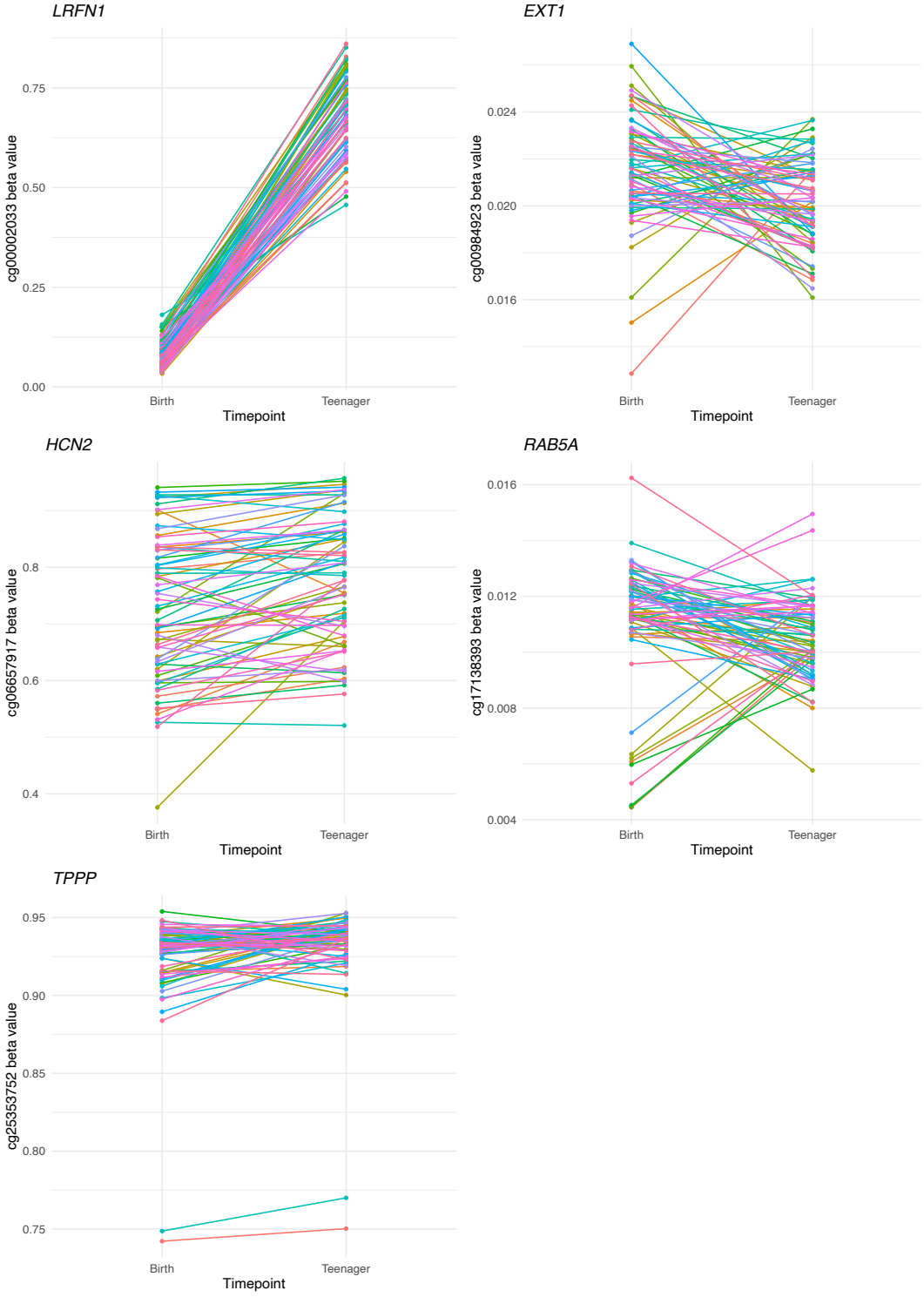


Figure 5.2: CpG-site-specific beta values (representing proportion methylated) from matched umbilical cord and venous blood at birth and adolescence. Each color and line represent an individually matched sample.



Note: y-axis varies by graph

Figure 5.3: *NINJ2* CpG-site-specific beta values (proportion methylated) from birth to adolescence. Each line represents an individual matched sample grouped by the SNP (C/G) rs34038797 (red=CC, green=GC, blue=GG, purple=not genotyped), a discovered methylation quantitative trait locus (MeQTL).

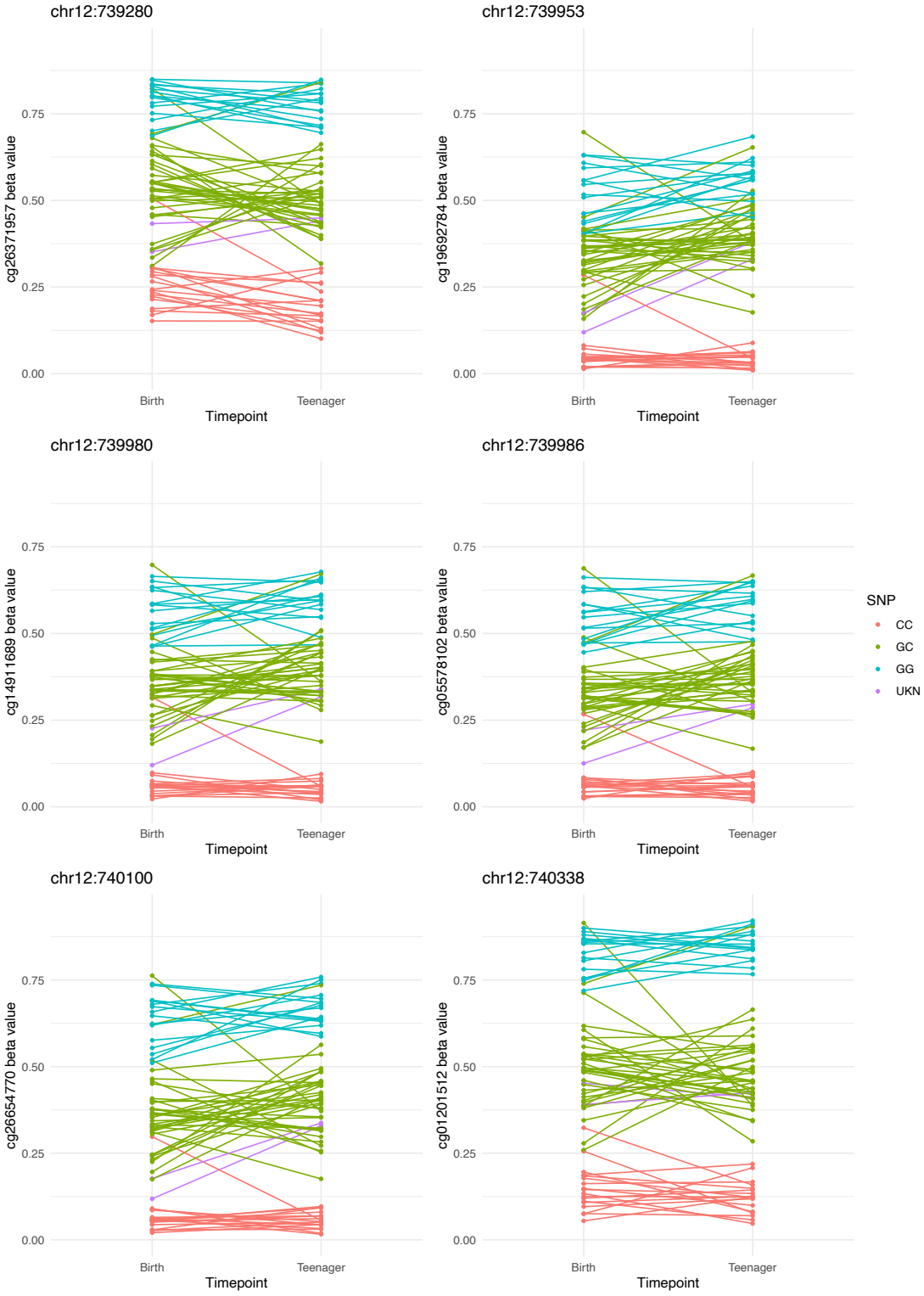
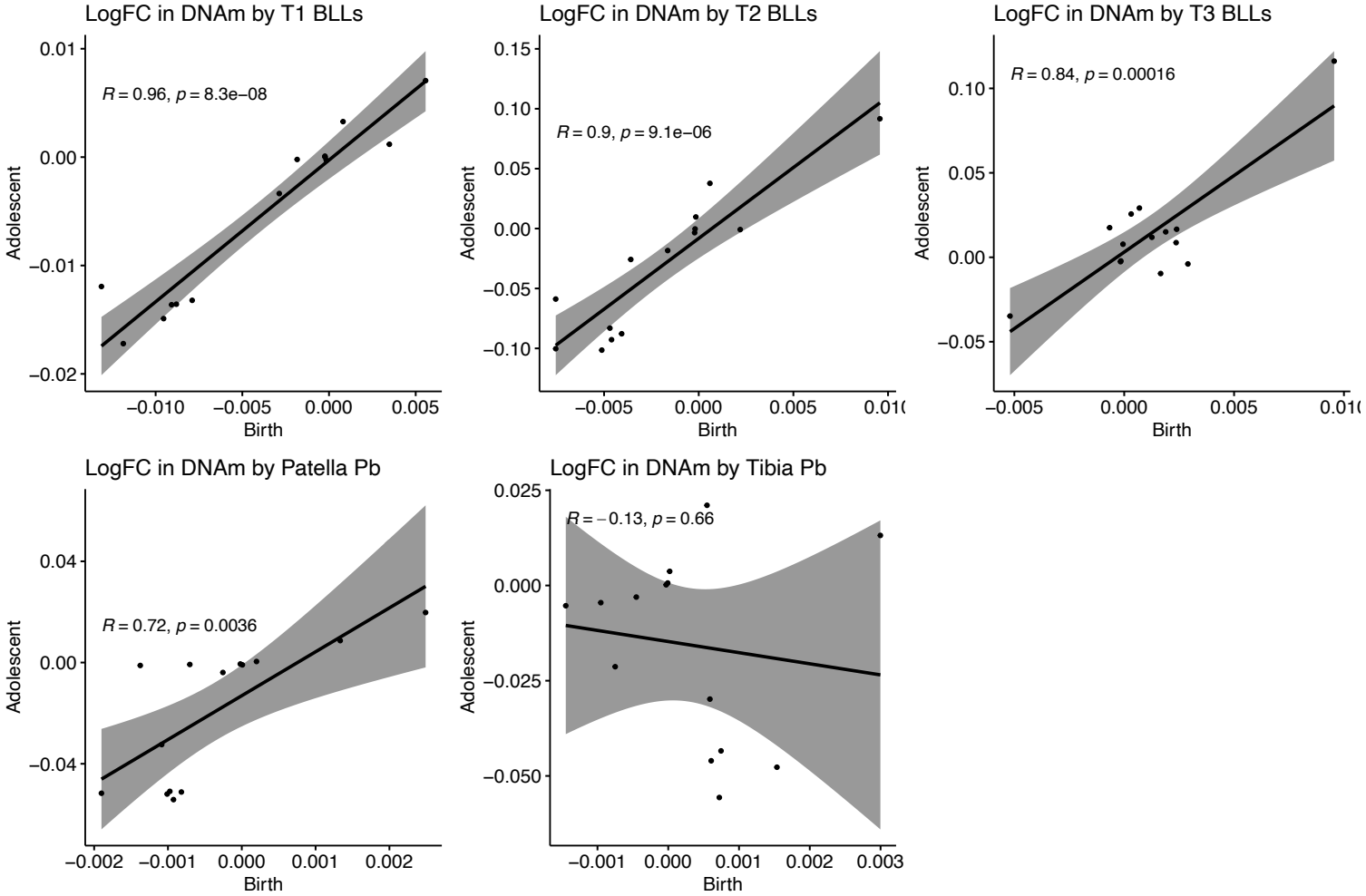


Figure 5.4: Correlation of beta effect estimates of change in DNA methylation (DNAm) per one-unit increase in prenatal Pb exposure for each biomarker at birth versus adolescence. Each dot represents the effect estimate for the association between the Pb biomarker and DNAm at each CpG-site at birth (x-axis) compared to the adolescent timepoint (y-axis). Black line is the correlation, where the grey shadow is the 95% confidence interval.



Note: CpG sites compared are cg17138393, cg00984923, cg25353752, cg26371957, cg19692784, cg14911689, cg05578102, cg26654770, cg01201512, cg06657917, cg03390844, cg01328348, cg00002033, cg034632088